The Dissertation Committee for Chi-Chun Fang Certifies that this is the approved version of the following Dissertation:

Embryogenesis, Trophic Eggs, and Early Colony Growth of Myrmicine Ants

Committee:

Ulrich G. Mueller, Supervisor

Lawrence E. Gilbert

Johann A. Hofmann

David S. Stein

Alexander L. Wild

Embryogenesis, Trophic Eggs, and Early Colony Growth of Myrmicine Ants

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Chi-Chun Fang

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Dedication

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Abstract

Embryogenesis, Trophic Eggs, and Early Colony Growth of Myrmicine Ants

Chi-Chun Fang, PhD

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Supervisor: Ulrich G. Mueller

Myrmicinae is the largest subfamily of ants (Formicidae, Hymenoptera) that encompass a great diversity of morphology, behavior, and life-histories. As model systems, ants have been used to address mainly questions of social cooperation, division of labor, and reproductive conflict, but little research has been accomplished to date on embryonic development of ants. In **Chapter 1**, I provide a brief summary of the current understanding of embryogenesis in insects, and specifically embryogenesis of ants. In **Chapter 2**, I use histology to characterize the embryonic development series of three myrmicine ant species, *Atta texana* and *Mycocepurus smithii* (both fungus-gardening ants), as well as *Solenopsis invicta* (red imported fire ant). These detailed developmental series of ant embryogenesis will be a foundation for future gene-regulatory studies of caste determination. I further study the gene expression patterns of two genes (*wingless* and *engrailed*) in *A. texana*, and I demonstrate that ants possess a mosaic embryogenesis combining features of both short and long germ-band development, which is different from the conventional long germ-band type development exhibited by the hymenopteran species *Apis* and *Nasonia*. In **Chapter 3**, I elucidate factors that optimize colony fitness

during the early nest-founding stage of the fungus-gardening ant *M. smithii*, a species that typically founds nests by single queens (monogyny), but mature nests have multiple queens (polygyny). By varying the queen-to-worker ratio in small experimental colonies set up with a standardized fungus-garden biomass, I identify factors contributing to colony fitness and gyne reproduction in *M. smithii*. In **Chapter 4**, I describe the discovery of a third kind of egg laid by *A. texana* foundresses in addition to reproductive and trophic eggs, and I report the first observation of nanitic males in *A. texana* that were killed by the foundress queen shortly after the males' eclosion. This dissertation investigates embryogenesis of three myrmicine ant species, the survival strategies of *M. smithii*, and a so-far undescribed type of trophic egg laid by *A. texana* foundresses. Ants represent a unique lineage with independently evolved developmental patterns that will contribute to future comparative studies of embryogenesis among social insects.

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Chapter 1: Introduction

Ants (Formicidae, Hymenoptera) are model systems for studying social biology because of their diverse morphology, behavior, and life-histories (Hölldobler and Wilson 1990; Bourke and Franks 1995; Kipyatkov and Lopatina 2015). Hymenoptera are diverse not only in species number, but also in mechanisms of advanced cooperation, specifically eusocial cooperation. Eusociality describes a specific colony organization in social species such as ants, bees, wasps, and termites (Batra 1966; Andersson 1984) characterized by: (1) reproductive division of labor; (2) overlap in the generations of cooperating individuals in the same nest; (3) cooperation to rear the brood; and (4) reproductive division between reproductive (sexual) individuals and non-reproductive (sterile) or subfertile workers (Batra 1966). Eusocial insects typically start a colony from a foundress queen, which can produce thousands or even millions of offspring in her life, and the colony is maintained by subfertile or non-reproductive workers. There are typically three morphological castes in a eusocial species: queen, worker and male. The exact ontogenetic mechanisms regulating development of queen versus worker morphologies are still largely unclear in ants. Except for Khila and Abouheif (2008, 2010) and Niculita (2006), little research on embryonic development has been accomplished on ants to date.

In eusocial Hymenoptera, every female egg is pluripotent and can follow different ontogenetic trajectories, to develop into either a queen or a worker (Bonasio et al. 2012); that is, with very few known exceptions (Clark et al. 2006), caste is not determined already at the egg stage, but sometimes during larval development, dependent on nutritional factors (Michener 1974; Hölldobler and Wilson 1990) or hormonal factors (Wheeler 1986; Penick et al. 2012). In addition, epigenetic mechanisms (imprinting by

mother queens) could potentially be critical factors in caste determination (Weiner and Toth 2012). Generally, female alates and workers (i.e., female offspring produced by a queen) have the same set of chromosomes but develop different phenotypes.

Exactly when and how queen/worker caste is determined in ants – at an early developmental stage or late larval instar stage – is still unclear (Wheeler 1986; Grbic et al. 1997; Khila and Abouheif 2008, 2010). The prevailing view is that caste determination in ants could occur at a late larval stage, under the control of the intrinsic endocrine system (Wheeler 1986; Grbic et al. 1997). Juvenile hormone (JH), for example, is a key hormone controlling development in all insects, and JH therefore also plays a key role in ants, especially influencing queen development. In the ant *H*arpegnathos *saltator*, for example, individuals treated with a JH analog (JHA) at the third and fourth instar larval stage developed into queens under laboratory conditions, whereas untreated larvae developed into workers (Penick et al. 2012). In addition to hormonal factors, environmental cues can have effects on caste regulation in social insects as well (Hölldobler and Wilson 1990; Wheeler 1994). For example, nutrient fed to larvae can influence caste differentiation (Richards and Packer 1994; Wheeler 1994), or the queen/worker ratio, which depends on colony size in many ants, can alter caste development (Boulay et al. 2009; Schmit et al. 2011; Ruel et al. 2012). Moreover, cues from eggs, larvae, and workers can influence caste differentiation. Workers of *Aphaenogaster senilis*, for example, are hypothesized to have more control on gyne development when the queen signal fades (Boulay et al. 2007). In *Monomorium pharaonis*, caste regulation is a colony-level collective behavior where the number of late-instar larvae limits gyne production, whereas worker production is limited by eggs (Warner et al. 2016). For example, the surface hydrocarbons of queen eggs will influence worker production in *Camponotous floridanus* (Endler et al. 2004). In summary,

therefore, different developmental stages or environmental factors are crucial in caste regulation, but other evidence suggests that caste could possibly be determined during early developmental stages regulated by little-understood early caste-formation processes (Khila and Abouheif 2008, 2010).

The best-studied insect developmental system is *Drosophila melanogaster* (Campos-Ortega and Hartenstein 1985; Small et al. 1991; Akam 1994; Amiri and Stein 2002). *Drosophila* embryos undergo a so-called *long germ-band* mode of development, in which the entire segmented anterior-posterior axis is established simultaneously, and in which the developing embryo fills the egg, with little or no increase in overall size occurring during embryogenesis (Akam 1994). More basal insect species undergo *intermediate* or *short germ-band* development, in which the developing embryo occupies a smaller portion of the egg, with more posterior segments forming progressively over the course of embryogenesis and in which an embryo grows gradually to fill the egg (Dearden and Akam 2001; Lynch and Roth 2011). Like *Drosophila*, other hymenopteran species that have been characterized (*Apis, Nasonia*) undergo long germ-band development (Lynch et al. 2012; Wilson et al. 2014).

To elucidate caste differentiation, it is possible to characterize gene expression at different developmental stages. According to Ometto et al. (2011), much of the evolution in gene expression in ants might have happened in the worker caste, rather than the queen caste (i.e., queens are transcriptomically more similar to solitary females of other hymenotperan lineages). In addition, pattern-formation genes may determine the developmental fate of caste at early stages of postembryonic development (Miura 2005). In honeybees, gene expression differs between queen and workers, and about 50 candidate genes determine whether a bee is a nurse or a forager (Wheeler 1986; Evans and Wheeler 1999; Whitfield et al. 2003). Gene expression can change temporally in

honeybee workers, for example when workers transition from nursing to foraging as they age (Ben-Shahar et al. 2002). Such behavioral transitions are related not only to gene expression, but also to environmental factors that can trigger specific behaviors.

To address the queen/worker caste determination question, I integrate in this dissertation research approaches from developmental biology, ecology, morphology and comparative biology. Specifically, I (1) explore ant embryonic development (i.e., embryogenesis), which has received far less attention compared to the ecology, phylogeny, and behavior of ants; (2) manipulate queen-worker-garden ratios in the fungus-growing ant *Mycocepurus smithii* to understand life-history strategies during the early nest-founding stage; and (3) describe a new type of ant trophic egg in the fungus-growing ant *Atta texana*.

I have been interested in the question of whether events during embryogenesis at the egg stage influence caste determination. Before addressing that question, it is necessary to characterize in detail the process of embryogenesis in ants. In Chapter 2, I characterize embryonic developmental patterns (i.e., embryogenesis) of three ant species in the subfamily Mymricinae, the two fungus-growing ant species *Atta texana* and *Mycocepurus smithii* (tribe Attini), as well as the fire ant *Solenopsis invicta* (tribe Solenopsidini). Surprisingly, embryogenesis in these three species does not show the same long-germ features found in two other hymenopteran species studies so far, the honeybee *Apis* and the parasitic wasp *Nasonia* (Lynch et al. 2012; Wilson et al. 2014; Cridge et al. 2017). Instead, developmental series (with DAPI staining) of the three studied ant species exhibit embryonic development that resembles corresponding features of the polyembryonic wasp *Macrocentrus cingulum* (short germ-band, Sucena et al. 2014) and features of *Schistocerca gregaria* (short germ-band, Dearden and Akam, 2001). In intermediate-germ embryos (e.g., *Tribolium*, *Oncopeltus*, *Acyrthosiphon*, and

Eusceli), the cell fate-map occupies about half of the whole embryo (Handel et al., 2005; Lynch and Roth, 2011). Short-germ embryos have the shortest length of germ band among these three types (e.g. *Gryllus*, *Schistocerca*, *Atrachya*, *Thermobia*) (Dearden and Akam, 2001; Lynch and Roth, 2011).

At early embryonic stages of the three studied ants species, the embryo occupies a small portion of the egg, and over the course of embryogenesis the embryo undergoes considerable growth. In additional experiments, I describe the expression of the segment polarity genes *wingless* and *engrailed* in the fungus-growing ant *A. texana*. Expression patterns of *wingless* and *engrailed* provide evidence of embryogenesis in the myrmicine ant *A. texana* that is different from long germ-band development. Embryogenesis in myrmicine ants exhibits features of both short and long germ-band development, which is surprising given prior expectations derived from studies of *Apis* and *Nasonia*. This discovery lays a foundation for future research on genetic regulation of embryogenesis, for example gene-regulatory events during embryogenesis involved in queen/worker caste determination.

In Chapter 3, I elucidate factors that optimize colony fitness during the early nest-founding stage of the fungus-growing ant *Mycocepurus smithii*, a species that typically founds nests monogynously (by single queens), but mature nests are polygynous (multiple queens). I conduct experiments to understand the transition from monogyny (i.e., single queen) to polygyny (i.e., multiple queens) under lab conditions. To understand factors that optimize colony fitness and garden growth in *M. smithii*, I vary the queen-to-worker ratio in small experimental colonies set up with a standardized fungus-garden biomass. The goals are to test (1) whether an incipient colony with multiple queens (pleometrosis) is able to grow and sustain a larger biomass of fungus garden than a single-queen colony (haplometrosis); (2) whether there exists an optimal

number of workers that an incipient colony can support with a given garden biomass; and (3) whether there exists a specific queen-to-worker ratio when colonies transition from monogyny to polygyny. Studying the founding colony fitness contributes to understanding of survival strategies in *M. smithii* and the specific factors of producing new gynes (i.e., daughter queens), such as queen-to-worker ratios or worker-to-garden ratios.

In Chapter 4, I describe a third kind of egg laid by *Atta* foundresses in addition to reproductive and trophic eggs in incipient nests. I use fluorescent microscopy to show that this third type of egg represents reproductive eggs that fail to develop and that are somewhat larger (≈ 490µm x 317µm) than regular reproductive eggs (≈ 425µm x 240µm), but smaller than trophic eggs (≈ 640 µm x 530µm). When not consumed by ant larvae, trophic eggs liquify by some endogenous process within 24 hours after oviposition to release nutrient contents. In contrast, undeveloped reproductive eggs do not liquify, but undeveloped reproductive eggs can be digested by the fungus, whereas reproductive eggs are not digested by the fungus to complete their development. I also report the first observation for *A. texana* of nanitic males (presumably diploid males) that were killed by the foundress queen shortly after the males' eclosion.

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Chapter 2: Embryogenesis in myrmicine ants exhibits features of both short and long germ-band development

INTRODUCTION

Insect embryogenesis is classified morphologically into three types, called long germ-band, intermediate germ-band, and short germ-band embryogenesis (Akam 1994; Davis and Patel 2002; Lynch and Roth 2011; Lynch et al. 2012). In embryos undergoing long germ-band embryogenesis (e.g., *Drosophila, Anopheles*, *Apis*, *Nasonia*, *Bombyx*), the fate-map of the portion of the egg that constitutes the embryonic primordium (the germ-band) encompasses almost the whole embryo, with the embryo already containing the undeveloped rudiments of the future head, thorax, and abdomen (Araujo and Bier 2000; Lynch et al. 2006). In contrast, the other two types of embryos initially develop only the domains of the future head, part of the thorax, and initially none of the abdominal segments. In intermediate germ-band embryos (e.g., *Tribolium*, *Oncopeltus*, *Acyrthosiphon*, and *Eusceli*), the cell fate-map comprises about half of the whole embryo (Handel et al. 2005; Lynch and Roth 2011). Short germ-band embryos have the shortest length of germ-band among these three types (e.g., *Gryllus*, *Schistocerca*, *Atrachya*, *Thermobia*) (Dearden and Akam 2001; Lynch and Roth 2011).

One approach to classifying the germ-band type of arthropod embryogenesis is to examine the expression of genes that regulate segmental development (Larsen et al. 2008; Swarup and Verheyen 2012). *Drosophila melanogaster* is the most thoroughly studied model species in such parasegmental boundary (i.e., a parasegment is the unit of a posterior body segment plus the next adjacent anterior body segments in the embryo) studies, including expression of conserved segmental polarity genes (e.g., *engrailed*, *wingless*, and *hedgehog*) that separate the anterior and posterior portions of each body segment (Fjose et al. 1985; Larsen et al. 2008; Swarup and Verheyen 2012). *Drosophila*

embryos exhibit a long germ-band mode of embryogenesis, in which cell specification occurs almost simultaneously along the entire segmented anterior-posterior axis. From the molecular perspectives, two conserved genes, *wingless* (*wg*) and *engrailed* (*en*), are expressed at each developing body segment to form fourteen parasegmental boundaries (Martinez-Arias and Lawrence 1985). Other insect species (e.g., *Gryllus, Tribolium*) undergo short or intermediate germ-band development, in which the developing embryo initially occupies a smaller portion of the egg, with more posterior segments forming progressively over the course of embryogenesis and with the embryo gradually growing to fill the egg. The parasegment boundaries become therefore established gradually with age (Choe et al. 2006; Choe and Brown 2009). Similar to *Drosophila*, some hymenopteran species (*Apis, Nasonia*) also show long germ-band development (Lynch et al. 2012; Wilson et al. 2014). In contrast, embryogenesis of a third hymenopteran species, the polyembryonic wasp *Macrocentrus cingulum*, exhibits features of short-germ development (Sucena et al. 2014). This indicates that species in the order Hymenoptera as a whole potentially exhibit a greater diversity in fate-map than traditionally assumed, compared to species studied so far in the orders Coleoptera (beetles such as *Tribolium*, intermediate germ-band) and Diptera (flies, such as *Drosophila* and *Anopheles*, long germ-band). The possibility of unknown diversity of fate-maps in the Hymenoptera motivated our study to characterize expression patterns of developmental genes in ants (Formicidae, Hymenoptera).

Ants are model systems for studying social biology because of their diverse morphology, behavior, and life-history traits (Hölldobler and Wilson 1990; Bourke and Franks 1995; Kipyatkov and Lopatina 2015). In ants, the formicoid clade is the largest clade that encompasses about 90% of all ant species and 14 of the 20 known subfamilies, including the species-rich subfamily Myrmicinae studied here (Ward 2007; Ward et al.

2015). We examine here *Atta texana*, *Mycocepurus smithii* and *Solenopsis invicta*. Although these three species belong to the same subfamily (Myrmicinae), they are behaviorally, ecologically, and genetically very distinct. For example, of the fungus-growing ants studied, *Atta* exhibits complex higher fungiculture with pronounced worker caste polymorphism, whereas *Mycocepurus* exhibits lower fungiculture with monomorphic workers (Schultz and Brady 2008; Mueller 2015; Mueller et al. 2017, 2018), making them good candidates for comparison of early developmental processes. Aside from Khila and Abouheif (2008, 2010) and Niculita (2006), little research on embryonic development has been accomplished on ants to date.

To characterize embryological development, we compile here images representing a complete chronological series of developmental stages ("developmental series") of embryogenesis for three myrmicine ants, the two fungus-growing ants *Atta texana* and *Mycocepurus smithii*, as well as the red imported fire ant *Solenopsis invicta* for comparison. For *A. texana*, we have also characterized the expression of two conserved genes (*wingless* and *engrailed*) that are known to be involved in embryonic segmentation (Martinez-Arias and Lawrence 1985; Dearden and Akam 2001; Choe et al. 2006). Integration of this morphological and genetic information of ant embryogenesis permits classification of the particular germ-band type exhibited by these ant species. Surprisingly, the three myrmicine ants studied do not show the typical long germ-band type of the two most closely related hymenopteran species for which germ-band type is known, the honeybee (*Apis*) and a parasitic wasp (*Nasonia*), but rather a germ-band type that includes features of both short and long germ-band development that is similar to the more distantly related polyembyonic wasp *Macrocentrus cingulum* (Sucena et al. 2014).

MATERIALS AND METHODS

Ant species

We studied embryo development in three ants of the subfamily Myrmicinae (Hymenoptera, Formicidae), two fungus-growing ants (the so-called the leafcutter ant *Atta texana*, and the primitive fungus-growing ant *Mycocepurus smithii*) and the red imported fire ant *Solenopsis invicta*.

Atta texana is a leaf-cutting fungus-growing ant. There are three different castes in *A. texana*: queen, male, and worker. Morphological castes in workers differ markedly in body size from small minims to large soldiers (Weber 1972; Wetterer 1999; Schultz and Brady 2008).

Mycocepurus smithii, also a fungus-growing ant, is an unusual ant species that reproduces asexually by thelytokous parthenogenesis (Himler et al. 2009; Rabeling et al. 2009, 2011; Rabeling and Kronauer 2013), where diploid female offspring develop from unfertilized eggs (Fernández-Marín et al. 2005; Himler et al. 2009). Rabeling et al. (2011) documented that sexual populations of *M. smithii* appear to exist in the Amazonas region of Brazil, but not in Central American populations where our lab colonies had been collected (e.g., no males have been found in our experimental clonal lineages kept in our lab since 2001). There exist therefore only two kinds of different caste size in *M. smithii* colony, queens and monomorphic workers.

Solenopsis invicta, the red imported fire ant, is an invasive species that can cause serious damage to agriculture and natural environments (Ascunce et al. 2011). Like *A. texana*, fire ant workers have morphological castes that differ in total body size (Wheeler 1991). Colonies of *S. invicta* exists in two forms, single-queen (monogyne) and multiple-

queen (polygyne) colonies (Ross and Fletcher 1985a). Worker size is generally smaller in polygyne colonies than the workers in monogyne colonies (Greenberg et al. 1985).

Colonies establishment and maintenance

M. smithii colonies were collected in central Panama in 2001 using the methods described in Kellner et al. (2013), then maintained as lab colonies using the methods of Sosa-Calvo et al. (2015). To establish mature lab colonies with 1-3 large gardens, *Atta texana* queens were collected in 2014 after a mating flight at Fort Belknap, Texas, then allowed to found lab colonies using the methods of Marti et al. (2015). To compare embryogenesis in these mature colonies with embryogenesis of newly founded colonies, we also collected *A. texana* foundresses on May 5th, 2018 after a mating flight at Commons Ford, Austin, Texas. Both fungus-growing ant species were given water *ad libitum* together with a mixture of polenta and finely ground oat flakes (ratio 3:1) as substrate for fungiculture. Attine colonies were kept in a temperature-controlled (but not humidity-controlled) laboratory room (22-24 °C; 12:12 [L: D]) in Patterson Laboratory at the University of Texas at Austin. *S. invicta* colonies were collected in 2017 from Ecolab properties in the Austin area. The colonies of *S. invicta* were maintained in a controlled environment (25 °C; 12:12 [L: D]; RH 60 ± 5%) in the Fire Ant Lab at Brackenridge Field Lab of the University of Texas at Austin. Each *S. invicta* colony was given *ad libitum* water, sugar water, and frozen crickets.

Embryo collection

Because the egg-laying capacities of queens of the three species are different, we used different methods for egg collection. For *M. smithii*, because one queen can produce only 1-2 eggs in a 24-hour period, we pooled 10 queens and transferred them to the same

Petri dish overnight for simultaneous egg collection. For *A. texana,* we placed a single queen into a round plastic container (5.5cm diameter; 3.7cm height) for egg collection, as described in detail by Marti et al. (2015). A typical young foundress of *A. texana* lays 5-15 eggs in a 24-hour period, while a typical mature queen can lay 50-100 eggs within 24 hours. For both attine ant species, we added five workers and a small fragment of fungus (3 mm^3) into the same container in which eggs were collected, to promote care of the eggs by the workers (attine workers transfer fresh-laid eggs to the fungal garden). We used a bottom-layer of 3% Agar as the substrate in the containers to maintain humidity, but the smooth, clear agar-bottom also allowed simple detection of any eggs. We added 15µl diluted ampicillin (10 mg/ml) on a small piece of filter paper (about 2 x 2 cm^2), which we placed on the agar plate during egg collection to minimize bacterial contamination of the fungus. For fire ants, we placed a single *S. invicta* queen, together with 5 workers, into a Petri dish with a bottom layer of 3% Agar as the substrate, as described above. Because *S. invicta* queens oviposit at a high rate, we collected freshly-laid eggs 4 hours after introduction of the queen to the dish.

Embryo fixation

We moistened a fine brush with distilled water, then placed the embryos into a custom-made meshed basket. The basket was constructed by annealing a metal mesh to the cut bottom-end of a 50ml Falcon tube. To permeabilize the chorion of the eggs, we left the eggs on the mesh, which was then placed on a Petri dish. 10 ml of 15% bleach, diluted in water was then poured into the petri dish, followed by gentle agitation of the eggs by pipetting the solution up and down for 2 minutes to wash the eggs. After permeabilization, we rinsed the eggs for 30 sec in distilled water to eliminate any residual bleach. The eggs were then treated with 0.3% Triton for 5 min on ice, then transferred in

their baskets to boiling 0.3% Triton for 20 sec. Eggs were then rinsed quickly in ice-cold 1x Phosphate Buffered Saline (PBS), then transferred into ice-cold 0.1% Tween-20 diluted with PBS (PBST) for 5 min. For more convenient manipulation, we then transferred the eggs to a 1.5 ml Eppendorf tube and rinsed them three times in 0.1% PBST. Following the third rinse, the eggs were incubated in 0.1% PBST on ice for 1 hour. To permeabilize vitelline and embryonic membranes, the eggs were treated with Proteinase K (20 µg/ml stock solution in ethanol, diluted 500X in 0.1% PBST) for 5 min, followed by immersion in 1x glycine (20mg/ml stock solution in EB buffer, diluted 10x in 0.1% PBST) for 5 min on ice. Eggs were then rinsed three times with PEM buffer (recipe for 250mL PEM buffer is 8.66g PIPES, 0.19g EGTA, and 0.03g $MgSO_4$ in distilled water). We moved the eggs into a new 1.5 ml Eppendorf tube with the fixation solution (400µl PEM, 60µl 37% formaldehyde, and 500µl Heptane), then rotated the samples at room temperature for 25 min under 180 rpm. After removing the post-fix solution, we had the eggs stay in 200µl distilled water for one minute. Finally, we washed the eggs three times in ice-cold methanol (MeOH). Fixed embryos were stored in MeOH at -20°C.

DAPI staining

To rehydrate the fixed samples, we treated the eggs under room temperature in 75%, 50%, and 25% MeOH (diluted with 0.1% PBST), each time for 5 minutes. Eggs were then rinsed 3 times with 0.1% PBST, and we applied a 10 min wash with 0.1% PBST to the eggs on the shaker (100 rpm) at room temperature. To ensure quality staining, we applied a second permeabilization to the eggs, as follows: proteinase K for 5 min; glycine for 5 min; 3 times 0.1% PBST rinse; post-fixation (135µl formaldehyde with 865µl 0.1% PBST) for 25 min; and again, glycine for 5 min. To wash off the post-

fixation solution, we rinsed the sample again three times for 5 min in 0.1% PBST. The eggs were then immersed in a DAPI solution (1uL DAPI with 999 µl 0.1% PBST) for 30 min, then washed with 0.1% PBST for 5 min. Eggs were mounted in 75% glycerol (diluted with 0.1% PBST), followed by incubating the eggs in a series of solutions of increasing proportions of glycerol diluted in 0.1% PBST as follows steps: 25% glycerol for 5 min; 50% glycerol for 30 min; 75% glycerol overnight at 4 °C.

Whole-mount *in situ* hybridization

We designed the *wingless* probe based on the sequence of that gene in the published genome of *Atta colombica* (Wnt-1 mRNA; NCBI reference sequence: XM_018204929.1). The entire *in situ* hybridization procedure was carried out over 3 days: Proteinase K digestion, post-fixation, and hybridization on Day 1; several steps for the washes on Day 2; and staining with NBT/BCIP on Day 3. All steps were performed in 1.5ml Eppendorf tubes placed on a rocker. In addition, we used 1ml buffer (or washing solution) at each step, unless otherwise indicated. Details of these three-day protocol are listed in appendix A and appendix B.

Whole-mount immunohistochemistry

To characterize the expression of the Engrailed (En) protein during segmentation, we treated the dissected embryos with monoclonal anti-engrailed (clone 4D9 from the Developmental Studies Hybridoma Bank, DSHB) as the primary antibody and goat anti-mouse (Alexa Flour 488) as the secondary antibody. The entire antibody staining procedure takes 2 days. On Day 1, rehydration of dissected embryos and post-fixation before treatment with the monoclonal primary antibody on Day 1; followed by overnight blocking of embryos in 5% normal goat serum (NGS) (diluted with 0.1% PBST), then

treated with 4D9 at 4 °C. Concentration for 4D9 was 5µg/µL (diluted 4D9 in 1% NGS; 1% NGS was diluted with 0.1% PBST). On Day 2, embryos were washed with 1% NGS (diluted with 0.1% PBST) 4 times at room temperature, then treatment with secondary antibody (diluted 1:750 in 1% NGS) for 2 hours at room temperature. This was followed by washing of embryos with PBST four times, for 15 minutes each. The embryos were then mounted for imaging as described above for DAPI staining.

Microscopy

Fluorescent-microscopy images were taken with a Zeiss Axiovert Fluorescent Light Microscope at the Microscopy and Imaging Facility at the University of Texas at Austin (http://sites.cns.utexas.edu/cbrs/microscopy). Images of *in situ* hybridization were taken by a Leica MZ16 Stereomicroscope fitted with a DFC420 digital camera; images of immunohistochemistry were taken by using a Nikon confocal microscope C2/C2si fitted with Ti2-LAPP Ti2 laser application system.

RESULTS

Embryogenesis of three myrmicine ants

At the onset of embryogenesis in *A. texana*, the first two nuclei arising from the first mitotic division of the fertilized zygote appear 14-18 hours after oviposition (Fig. 2.8). For an egg laid by a foundress queen, the blastoderm stage begins on Day 2 (Fig. 2.1) when a single layer of embryonic epithelial tissue formed prior to the formation of body axes (i.e., multiple nuclei could be seen distributed within the developing tissue). On Day 3, the embryonic primordium forms as a heart-shaped germ-band at the posterior end, followed by elongation of the germ-band. On Day 4, gastrulation occurs (Fig. 2.9C). The period of embryo extension (elongation) lasts from Day 4 to Day 7. At the end of the

elongation phase, the future head lobe bulges out on Day 8, followed by morphological signs of sequential segmentation appearing along the anterior-posterior axis. On Day 9, the three gnathal segments, labeled mandibular (md), maxillary (mx), and labial (lb), develop (Fig. 2.1Day9 & 2.7A). On Day 10, the future thoracic region starts to form, and the three distinguishable future thoracic segments develop completely on Day 11. After development of the thoracic segments, the morphologically distinct abdominal segments appear sequentially on Day 12. On Days 13 and 14, germ-band retraction proceeds gradually. On Day 15, the 1st-instar larva hatches (at 25 ± 1 °C) (Fig. 2.1) to complete embryogenesis of eggs laid by a foundress queen within 15 days. In contrast, complete embryogenesis of eggs laid by a mature queen takes 20 days (see details below).

In *Mycocepurus smithii*, embryogenesis lasts 19-20 days at 25 ± 1 °C (Fig. 2.2). The first mitotic division is observed within 24 hours and the blastoderm stage begins on Day 2. Between Day 3 to 5, the germ-band forms gradually towards to the posterior end of the embryo, and a heart-shaped germ-band has formed completely on Day 5 (Fig. 2.2). Germ-band elongation extends from Day 6 to Day 10. Meanwhile, the first segment (i.e., future head lobe) observed on Day 9, and the germ-band starts to curve from the lateral side and extend towards to the posterior side. On Day 10, three gnathal segments develop at the anterior part of the germ-band. From Day 11 to Day 15, the future thoracic and abdominal segments appear sequentially. Germ-band condensation (retraction) occurs between Day 16 to Day 19. Between Day 19-20, the 1st-instar larva hatches (at 25 ± 1 °C) (Fig. 2.2).

The egg stage of *Solenopsis invicta* lasts 9-10 days at 25 ± 1 °C (Fig. 2.3). The blastoderm appears on Day 2. From Day 2 to Day 3, the germ-band forms and extends rapidly. The future head lobe forms on Day 4, and the rest of the future body segments

develop on Day 5. The process of embryonic condensation extends from Day 6 to Day 8. Between Day 9-10, a 1st-instar larva hatches (at 25 ± 1 °C) (Fig. 2.3).

wingless (*wg*) mRNA expression

To study the process of segmentation in *A. texana*, we examined *wingless* (*wg*) mRNA expression in eggs laid by a mature queen, from Day 9 to Day 12 after oviposition (Fig. 2.4). *Wingless* is one of the conserved genes that is involved in insect segmentation (Swarup and Verheyen 2012). During the segmentation of *A. texana*, *wg* mRNA was expressed diffusely at both anterior and posterior ends of the embryo (Fig. 2.4A). At the time at which *wg* expression became more pronounced at the anterior of the embryo, the future head lobe formed (Fig. 2.4B). In addition, three condensed bands of *wg* mRNA could be detected on Day 10, which led to the formation of the three future gnathal segments (Fig. 2.4B, see arrows). The expression of condensed *wg* bands appeared sequentially on Day 10.5 with 9 bands (Fig. 2.4C) and Day 11 with 10 bands (Fig. 2.4D), followed by the formation of the abdominal segments. On Day 12, 12 *wg* bands were observed (Fig. 2.4E), suggesting the bands were being specified progressively over time during embryogenesis (i.e., all bands did not form at the same time).

Engrailed (En) protein expression

We also examined Engrailed (En) protein expression in eggs laid by a mature queen from Day 9.5 to Day 11 after oviposition (Fig. 2.5). *Engrailed* is another conserved gene, the product of which regulates the polarity of each body segment (Choe et al. 2006; Dearden and Akam 2001). During segmentation, 3, 8, and 13 condensed bands appeared on Day 9.5, Day 10 and Day 11, respectively, indicating that the segments were sequentially specified over time.

Comparative morphology of *Atta texana* foundress-laid egg and mature-queen-laid egg

We timed the development of eggs laid by foundress queens and mature queens. At the same temperature (at 25 ± 1 °C), embryos in eggs laid by the foundresses developed 25% faster than the embryos produced by mature queens (Fig. 2.6). For the foundress-laid eggs and mature queen-laid eggs, the developmental time was 15 and 20 days, respectively, from oviposition to hatching. To further characterize developmental trajectories between the two types of eggs, we examined the blastoderm, heart-shaped, elongation, and segmentation stage, which represent the four embryonic developmental landmarks during ant embryogenesis. There were no apparent differences at the blastoderm (Day 2) and heart-shaped stages (Day 3) between these two types of eggs. However, a developmental acceleration of foundress-laid eggs occurred at the elongation and segmentation stages. Embryonic elongation occurred at Day 4 in foundress-laid eggs, but on Day 6 in eggs laid by mature queens. In addition, segmentation started on Day 8 in foundress-laid eggs, but did not start until Day 10-11 in the mature queen-laid eggs (Fig. 2.6).

Comparing the two types of eggs on Day 9, a foundress-laid egg showed a well-developed future head lobe, as well as mandibular (md), maxillary (mx), and labial (lb) gnathal segments (Fig. 2.7A). In contrast, on Day 9 in the mature queen-laid egg, the future head was not fully developed, and the three gnathal segments had not started to develop (Fig. 2.7B). On Day 12, foundress-laid eggs showed the three well-developed segments of the future thorax (Fig. 2.7C), but there was no obvious morphological thoracic segmentation in the mature-queen-laid eggs on Day 12. We therefore examined *wingless* (*wg*) mRNA expressions in 12-day-old mature-queen-laid eggs, and discovered

three expression bands (Fig. 2.7D, see arrows), corroborating that thoracic segmentation occurred.

DISCUSSION

Our study aimed to characterize the embryonic development of three ant species in the subfamily Myrmicinae, two fungus-gardening ants (the leaf-cutter ant *Atta texana* and a so-called primitive fungus-growing ant *Mycocepurus smithii*), and the red imported fire ant *Solenopsis invicta*. Surprisingly, the ant embryos did not show the typical long germ-band developmental features seen in the previously examined hymenopteran species *Apis* and *Nasonia* (Cridge et al. 2017; Lynch et al. 2012; Wilson et al. 2014). Instead, complete developmental series representing stages of embryogenesis from oviposition to hatching in the three studied ant species (Figs. 2.1-2.3) exhibit embryonic patterns of development with some features that are observed in insects undergoing short and intermediate germ-band types of development, similar to what has been observed in the polyembryonic wasp *Macrocentrus cingulum* (Sucena et al. 2014).

Type of germ-band in ants

Length of the germ-band, which constitutes the embryonic primordium, is a useful way to characterize the pattern of embryogenesis occurring within groups of insects (Davis and Patel 2002). Unlike the long germ-bands seen in *Drosophila*, *Apis* and *Nasonia*, short or intermediate germ-bands embryos comprise only part of the egg volume at the beginning of development (e.g., *Tribolium*, *Gryllus* and *Schistocerca*) (Dearden and Akam 2001; Handel et al. 2005). Interestingly, insect oogenesis is correlated with these types of insect embryogenesis. Three kinds of insect oogenesis can be distinguished based on ovariole structure, called 1) polytrophic meroistic, 2)

telotrophic meroistic, and 3) panoistic ovaries (McLaughlin and Bratu 2015). Polytrophic meroistic ovaries are found mostly in long germ-band species, but there also exist a few short germ-band species with polytrophic meroistic ovaries (Lynch and Roth 2011). In our study, we discovered that the germ-bands of three myrmicine ants grow with time (Figs. 2.1-2.3), a feature that is unlike what is observed in canonical long germ-band insects. Interestingly, the ovaries of the three myrmicine ants studied are polytrophic meroistic (Fang personal observation), as in other ant species (Khila and Abouheif 2008, 2010). Thus, the association between polytrophic meroistic ovaries and canonical long germ-band development observed in *Apis*, *Nasonia*, and *Drosophila* is not conserved in the ant species that we have studied.

Atta texana (leaf-cutter fungiculture) and *Mycocepurus smithii* (so-called primitive fungiculture) represent, respectively, derived and early-branching lineages among fungus-growing (attine) ants, exhibiting distinct behavioral, ecological, and morphological features (Mueller et al. 2001; Schultz and Brady 2008), such as absence of worker polymorphism in *M. smithii* (all workers are morphologically similar), but workers show extreme worker polymorphism in *A. texana* (workers range from extremely small to large in the case of soldiers). Despite these differences at the egg stage, both attine species have a similar pattern of development lasting 19-20 days at 25 ± 1°C. Specifically, the timing of key developmental benchmarks (e.g., blastoderm, heart-shaped, elongation, segmentation and condensation) is similar during embryogenesis (Figs. 2.1, 2.2, and 2.9). For comparison, we also included in our study the fire ant, *Solenopsis invicta*, a species that belongs to the tribe Solenopsidini, which is sister to the tribe Attini that includes the fungus-growing ants (Ward et al. 2015). *S. invicta* has a shorter egg stage (9-10 days at 25 ± 1°C), compared to the two attine ants (19-20 days at 25 ± 1°C). The similarity in key developmental benchmarks (e.g., blastoderm, heart-

shaped, elongation, and segmentation; Fig. 2.9) between the two closely-related attine ants with their different worker polymorphisms suggests that embryonic development may be conserved in the relatively diverse ant clade Attini. When comparing *Solenopsis* with the two fungus-growing ants, we also can see the similar developmental benchmarks of blastoderm, elongation, and segmentation (Fig. 2.3). However, due to the faster development of fire ant embryos, we have captured the stage when the future germ-band nuclei migrated toward the posterior end of the egg but have not yet observed a heart-shaped germ-band (Fig. 2.3 Day2.5). Embryonic development of three myrmicine ants is therefore overall similar, but requiring different number of days for completion, indicating that some aspects of embryonic development are variable and not conserved within the subfamily Myrmicinae. Characterizing developmental pattern in additional ant lineages should generate further insights into the evolutionary-developmental bases underlying ant diversity.

To pattern of expression of the pair-rule gene *even-skipped* at the onset of gastrulation is an important feature for the classification of germ-band type during insect embryogenesis (Akam 1994; Patel et al. 1992). In *Drosophila*, seven pair-rule stripes of the *even-skipped* protein contribute to the specification of the segmental primordia of the future larval body plan. This occurs prior to gastrulation. However, in intermediate/short germ-band species, the initial expression of *even-skipped* protein at gastrulation exhibits fewer stripes than in the *Drosophila* embryo (Akam 1994; Davis and Patel 2002). Furthermore, *even-skipped* is involved in the initiation of *engrailed* striped expression (Patel et al. 1992). Consequently, with fewer expression stripes of *even-skipped*, one would expect to observe the initial formation of fewer expression stripes of *wingless* and *engrailed* during embryogenesis, as is true in *Tribolium* (Choe and Brown 2009) and *Schistocerca* (Dearden and Akam 2001). Gene expression of *wingless* and *engrailed*

during embryonic segmentation are therefore an additional criterion for classifying insect embryos (Cabrera et al. 1987; Dearden and Akam 2001; Swarup and Verheyen 2012; Vellutini and Hejnol 2016). In *A. texana*, although the germ-band in ants grows with time (Fig. 2.1), we observed striped expression of *wg*/*en* on Day 9 when the germ-band was already fully extended (Fig. 2.4 & 2.5). The embryogenesis pattern of *A. texana*, therefore, is different from that of other species undergoing a typical short germ-band pattern of development where the thorax/abdominal segments appear gradually during segmentation (Akam 1994; Choe et al. 2006; Davis and Patel 2002; Strobl and Stelzer 2014). Ants therefore possess a mosaic embryogenesis combining features of both short and long germ-band development.

Gastrulation seems to occur earlier than segmentation in the three studied ants (Fig. 2.1 Day4; Fig. 2.2 Day5; Fig. 2.3 Day3), which also differ from the process of segmentation in *Drosophila* where the seven striped *even-skipped* protein are fully expressed prior to gastrulation (Akam 1994). Characterizing expression patterns of conserved genes involved in gastrulation (e.g., *twist*; Baylies and Batel 1996) are needed to more precisely determine the timing of gastrulation versus segmental specification in ants.

During the segmentation stage of *A. texana*, the number of bands visible for *wingless* or for *engrailed* increases progressively with time, consistent with short or intermediate germ-band development and correlating with the progressive appearance of morphologically visible appearance of segments (Fig. 2.4 & 2.5). The overall development pattern therefore resembles the features found in segmentation of a polyembryonic wasp, *Macrocentrus cingulum* (Sucena et al. 2014). In *Drosophila*, body segments form simultaneously along the entire embryo, and the striped expression of *wingless* and of *engrailed* appears almost simultaneously in the germ-band extension

phase (Davis and Patel 2002; Hughes and Kaufman 2002). To understand the timing of *en*/*wg* expressions more closely, we searched images available at FlyPress 7, a database of *in situ* hybridization images in *Drosophila* (Kumar et al. 2017). Surprisingly, some *in situ* images of *Drosophila* embryos at stage 4-6 showed fewer than 14 stripes at the beginning of *wingless* expression (image id: FBim9038062; image Source: BDGP - insitu24864) and *engrailed* expression (image id: FBim9037015, image source: BDGP - insitu20703), suggesting that the posterior stripes of expression of these two segmentation genes appear somewhat later than the more anterior stripes. The features of non-simultaneous striped expressions of *wingless* and *engrailed* that we observed in ants are therefore somewhat similar to what is observed in *Drosophila*. In sum, the existence of short-germ features in a polyembryonic wasp and in the tree myrmicine ant species studies here, as well as the existence of long-germ features of two other hymenopteran species studied to date (*Apis* or *Nasonia*), indicates that the generation of the embryonic fate-map within the Hymenoptera is more diverse than expected.

Acceleration of development in eggs laid by foundress queens

The fitness strategy for a foundress queen is to maximize survival and brood production during a short period when workers are absent (Lotka 1925; Marti et al. 2015). Foundress queens are therefore typically time constrained (i.e., the queen needs to produce her first workers fast) and nutritionally constrained (i.e., the queen has limited resources at her disposal to produce the first workers). For example, in the paper wasp, *Polistes fuscatus*, foundress queens reduce the size of first-emerging workers, which consequently developed faster to help maintaining the colony (Reeve et al. 1998). Ant queens typically also produce smaller first workers, so-called nanitic workers (Espadaler and Rey, 2001; Porter and Tschinkel 1986; Tschinkel 1988), to accelerate development of

the first cohort of workers. Moreover, in the black garden ant, *Lasius niger*, the foundress takes advantage of higher temperatures to accelerate development of the first brood (Kipyatkov et al. 2004). Because nanitic workers occur also in leafcutter ants (Wetterer 1994; Wilson 1980), we compared the timing of embryogenesis of *A. texana* foundress-laid eggs and mature-queen-laid eggs. Foundress-laid eggs needed only 75% of the total time to complete embryogenesis compared to eggs laid by mature queens (Fig. 2.6). Moreover, the smaller size of nanitic workers presumably requires fewer somatic cells, consequently decreasing the time needed to complete the elongation and segmentation stages.

The mechanism underlying the developmental acceleration of foundress-laid eggs remains unknown. According to Ometto et al. (2011), much of the evolution in gene expression in ants appears to have happened in the worker caste, rather than in the queen caste, because queens are transciptomically more similar to females of solitary hymenopteran lineages. Specifically, the fast-developing eggs could express more age-associated senescence genes (Lee et al. 2010), for example, genes with pleiotropic effects that accelerate development at the nest founding stage, but have fitness costs later, but increasing initial colony growth, performance, and thus fitness of the incipient colony. In addition, pattern-formation genes may determine the developmental fate of caste in early stages of postembryonic development (Miura 2005). In fully developed adult honeybees, gene expression differs between queen and workers, and about 50 candidate genes determine whether a bee is a nurse or a forager (Wheeler 1986; Evans and Wheeler 1999; Whitfield et al. 2003). It is possible therefore that gene expression can change temporally in the first brood of ant workers during embryogenesis. Such transitions are related not only to gene expression, but also to environmental factors that can trigger specific behaviors.

Implications for understanding evolution of embryogenesis and caste determination

Among the four extant groups of arthropods (i.e., insects, crustaceans, myriapods, and chelicerates), only insects show a great diversity ranging from short to long germ-band segmentation (Damen 2007), whereas the other three arthropod lineages show only short germ-band segmentation. This has been traditionally interpreted that segmentation of the last common ancestor of these four arthropod lineages had short germ-band embryogenesis, and that a diversity of embryonic fate-maps evolved later within the insects (Damen 2007). If so, hemimetabolous insects such as *Gryllus* and *Schistocerca* would then have retained an ancestral state of short germband embryogenesis, and the diversity of germ-band types evolved later among holometabolous insects, such as Coleoptera (e.g., *Tribolium* with short/intermediate germ-band) Hymenoptera (e.g., *Apis*, *Nasonia* with long germ-band), Lepidoptera (e.g., *Bombyx* with long germ-band), and Diptera (e.g., *Drosophila* with long germ-band) (Davis and Patel 2002; Misof et al. 2014; Cridge et al. 2017). It is perhaps therefore not surprising that we found a germ-band pattern in the three myrmicine ants that is different from *Apis* and *Nasonia*, but similar to the polyembryonic wasp *Macrocentrus cingulum* (Sucena et al. 2014).

In addition to the high morphological and taxonomic diversity in the Hymenoptera (Johnson et al. 2013), there exists great variation in life-history between hymenopteran lineages, and it is therefore possible that the different germ-band types observed across ants, bees, and wasps may have been selected as embryological adaptations contributing to life-history traits (e.g. life span) or other specializations (e.g., parasitism; solitary versus social life). Characterizing and understanding such correlations between insect life history, specializations, and germ-band types will be a fruitful area for future research.

In eusocial Hymenoptera, every female egg can follow different ontogenetic trajectories, for example to develop into a queen or a worker (Bonasio et al. 2012). That is, with very few known exceptions (Clark et al. 2006), caste is not determined already at the egg stage. Factors other than DNA-sequence information, such as epigenetic mechanisms and environmental factors (e.g., nutrients), therefore must be critical factors in caste determination (Weiner and Toth 2012). In ants, the exact ontogenetic mechanisms underlying queen and worker determination are still unknown. One important hypothesis of queen-worker caste determination (Khila and Abouheif 2010) posits that the caste determination pathway could happen during oogenesis or early embryogenesis. Characterizing the gene regulatory networks (GRNs) during oogenesis and embryogenesis will therefore be necessary to elucidate the genetic toolkit underlying caste differentiation and sociality (Kocher 2018). Towards this future goal, our study identifies the germ-band type of three myrmicine ants, as well as developmental embryogenetic time series as a foundation for such future genetic and molecular studies of castes and sociality in insects.

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FIGURES

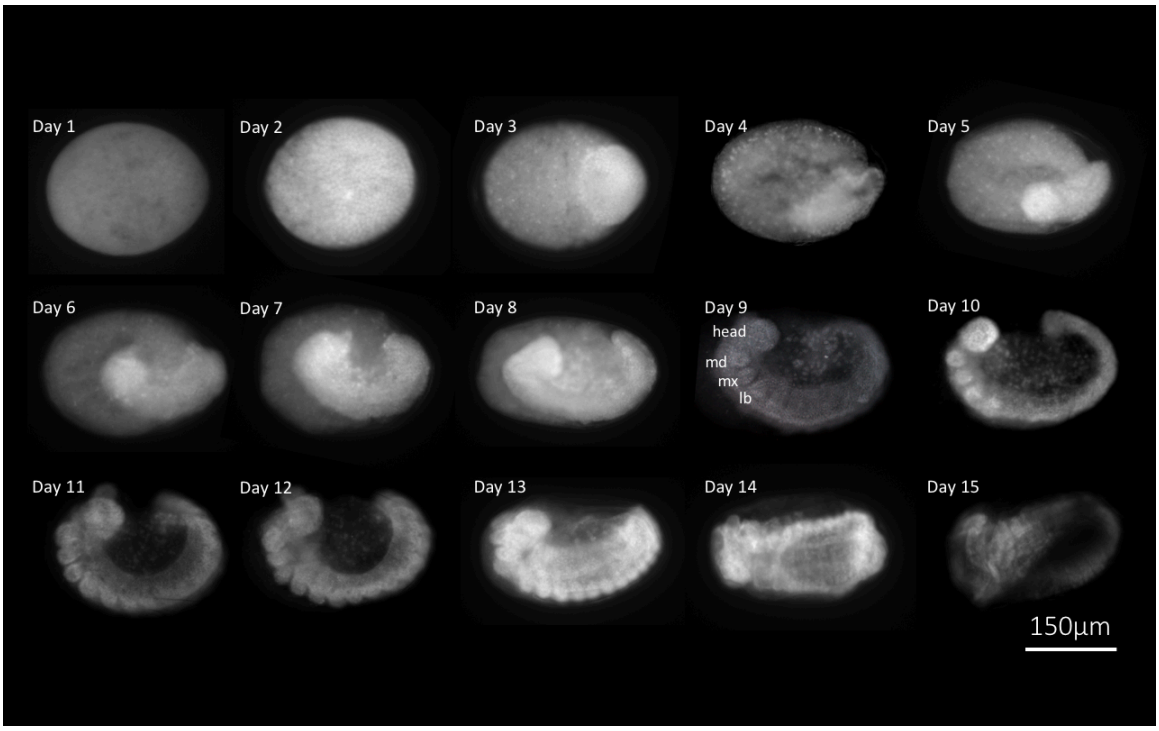

Figure 2.1. Developmental series of foundress-laid eggs of *Atta texana*, covering Day 1 to Day 15. Developing embryos in the eggs are stained with DAPI. The blastoderm stage occurs 24-48 hours after oviposition. A heart-shaped germ-band develops on Day 3. The germ-band elongates on Days 4-8, and the first segment (future head lobe) appears on Day 8. A clear separation of the future head and gnathal segments develops on Day 9; the three gnathal segments developing on Day 9 are the mandibular (md), maxillary (mx), and labial (lb) segments. The total egg stage for foundress-laid eggs lasts 15 days at 25 ± 1 °C, ending with hatching of the 1st-instar larva on Day 15; whereas the total egg stage for eggs laid by mature eggs is slower and lasts 20 days (see Fig. 2.6).

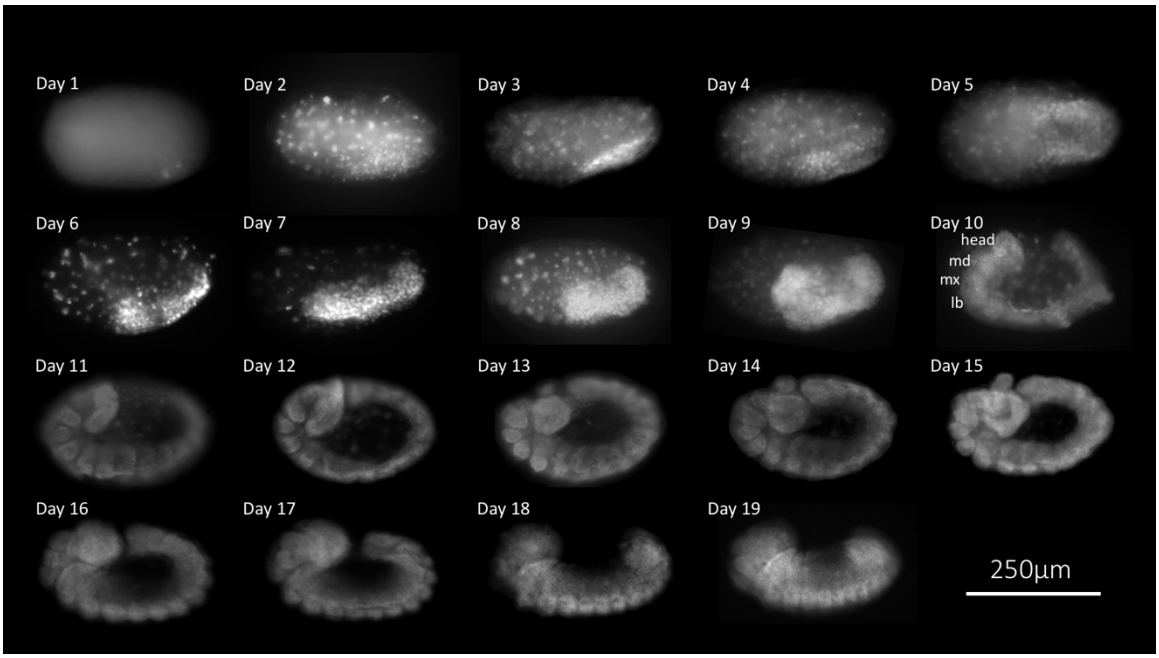

Figure 2.2. Developmental series of *Mycocepurus smithii*, covering Day 1 to Day 19. Developing embryos in the eggs are stained with DAPI. The blastoderm stage occurs 24-48 hours after oviposition. A heart-shaped germ-band develops on Day 5. The germ-band elongates on Days 6-10, and the first segment (future head lobe) appears on Day 9. A clear separation of the future head and the three gnathal segments (i.e., mandibular (md), maxillary (mx), and labial (lb)) develop on Day 10. Thoracic and abdominal segments form sequentially and increase in number from Day 11 to Day 15. Embryonic condensation occurs from Day 16 to Day 18. The total egg stage lasts 19-20 days at 25 ± 1 °C, ending with hatching of the 1st-instar larva on Day 19-20.

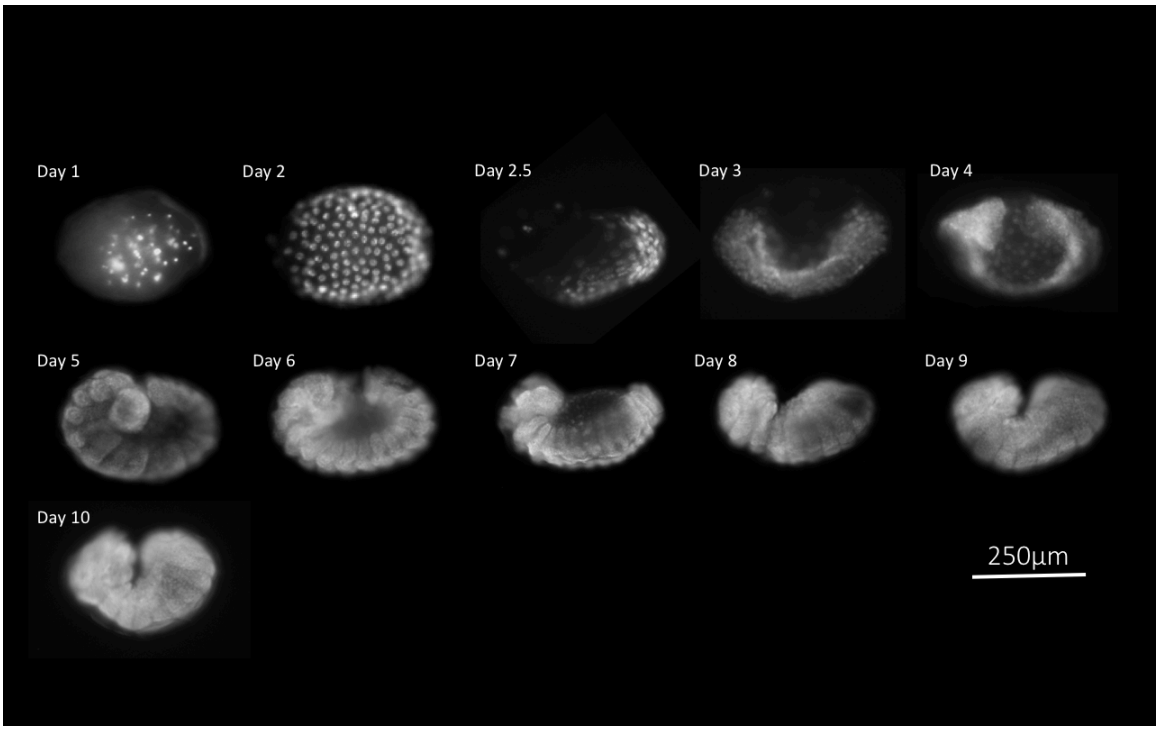

Figure 2.3. Developmental series of *Solenopsis invicta*, covering Day 1 to Day 10. Developing embryos in the eggs are stained with DAPI. The blastoderm stage occurs 24-48 hours after oviposition. The germ-band elongates on Day 2-4. The first segment (future head lobe) appears on Day 4, and segmentation happens on Day 4-5. The germ-band condensation starts on Day 7-9. The total egg stage lasts 9-10 days at 25 ± 1 °C, ending with hatching of the 1st-instar larva on Day 10.

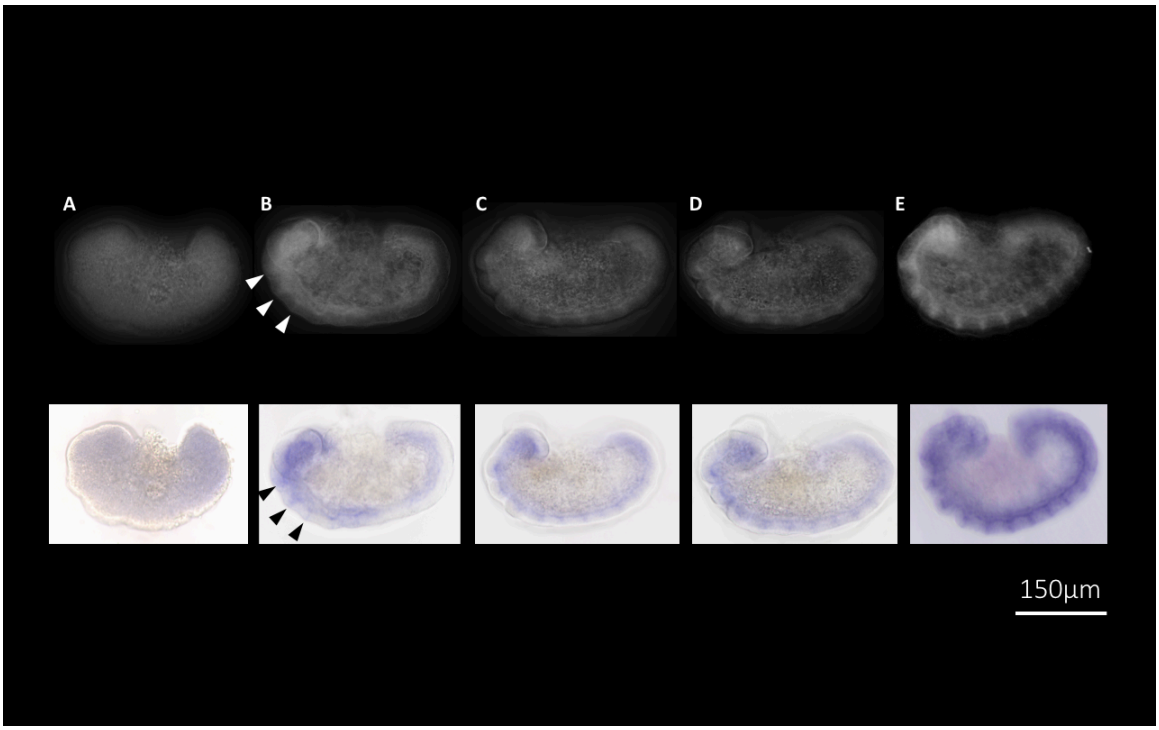

Figure 2.4. *In situ* hybridization series of *wingless* (*wg*) mRNA expression in eggs of *Atta texana* from Day 9 to Day 12 after oviposition. The eggs were collected from a mature *Atta texana* queen and were maintained at 25 ± 1 °C. The eggs are oriented to show the anterior on the left. Weak expression of *wg* mRNA appears on Day 9 (A) at both anterior and posterior ends of the embryo. The number of condensed bands of *wg* signal increases every additional 24 hours, showing 4 bands on Day 10 (B), 9 bands on Day 10.5, (C) 10 bands on Day 11 (D), and 12 bands on Day 12 (E).

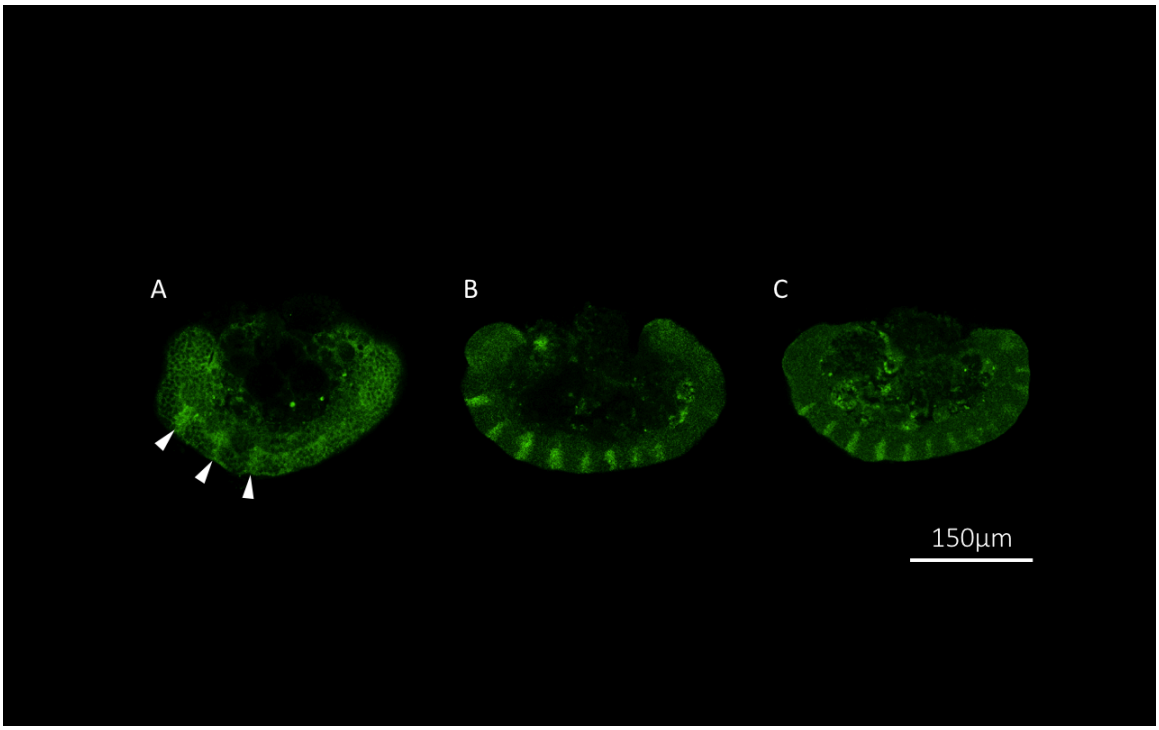

Figure 2.5. Immunohistochemistry series of *engrailed* (En) protein expression in eggs of *Atta texana* on Day 9.5 (A), Day 10 (B), and Day 11 (C) after oviposistion. The eggs were collected from a mature *Atta texana* queen and were maintained at 25 ± 1 °C. The eggs are oriented to show the anterior on the left. The number of segments in which engrailed was expressed increases with time. On Day 9.5 (A), three condensed bands appeared (see arrows); on Day 10 (B) 8 condensed bands appeared; and 13 bands on Day 11 (C).

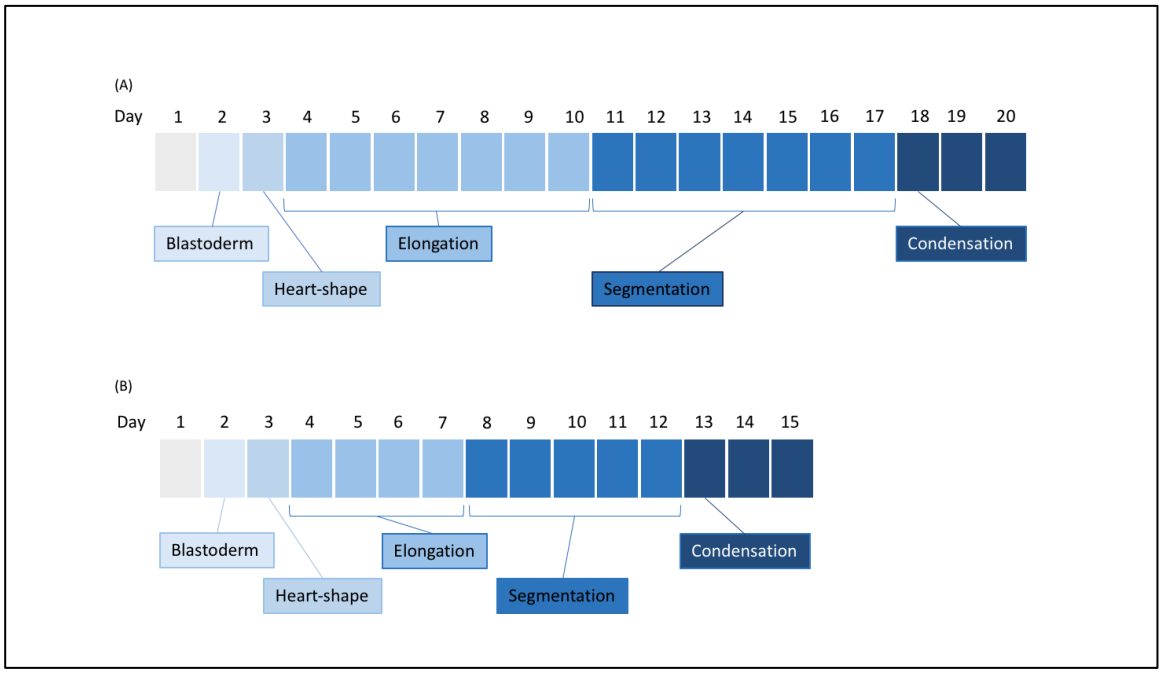

Figure 2.6. Developmental trajectories of (A) mature queen-laid eggs and (B) foundress-laid eggs of *Atta texana* at 25 ± 1 °C. Eggs laid by a foundress queen develop 25% faster than eggs laid by a mature queen, respectively needing 15 days versus 20 days for development from oviposition to hatching of the 1st-instar larva. Blastoderm development occurs on Day 2 in both types of eggs, but subsequent development is accelerated for eggs laid by a foundress queen. Among the four embryonic developmental stages, the acceleration affects the onset of elongation (Day 4 versus Day 6 in eggs laid, respectively, by foundress versus mature queens) and the onset of segmentation (Day 8 versus Day 11).

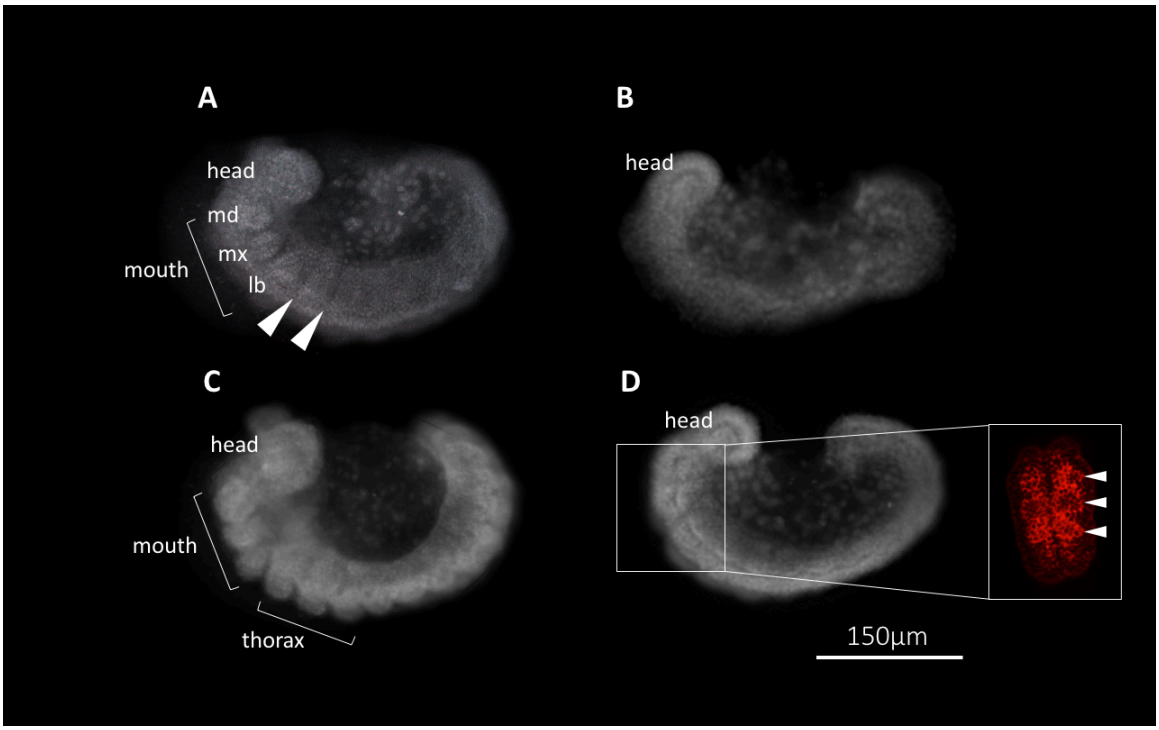

Figure 2.7. Comparative morphology of 9-day-old embryos produced by an *Atta texana* foundress queen (A) and a mature queen (B), as well as 12-day-old embryos produced by an *Atta texana* foundress queen (C) and a mature queen (D). The eggs are oriented to show the anterior on the left and dorsal at the top. In an egg of a fast-developing nanitic worker laid by a foundress queen (A), the future head lobe and thorax segments (see three arrows in A) are already formed by Day 9. In contrast, an egg of a regular worker laid by a mature queen shows on Day 9 only a partial development of the future head lobe, and no thoracic segmentation. (C) Formation of the future head lobe and 14 body segments. (D) Formation of a well-developed future head lobe and 3 gnathal segments. Expression of *wingless* (*wg*) (see arrows in D) was observed during the formations of future gnathal (mouth) segments. The three gnathal lobes were not developed completely at this stage, but *wg* expression signal had already appeared before the segments formed completely. The mRNA staining image was taken from ventral/anterior aspect of the embryo.

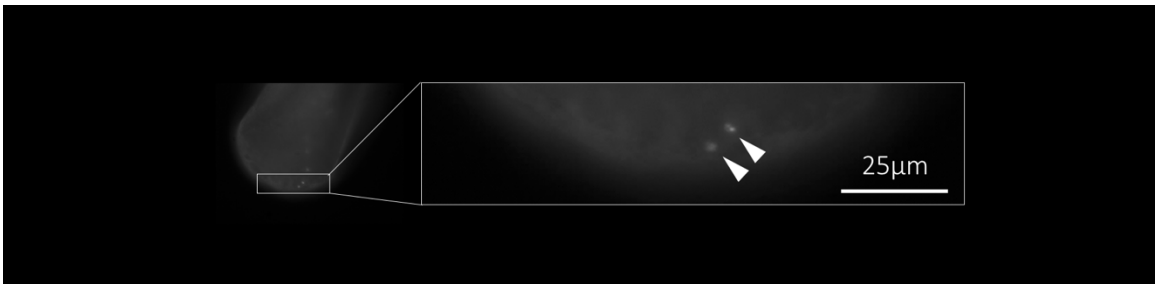

Figure 2.8. The first two nuclei stemming from the first division appeared 14-18 hours after oviposition by a mature *Atta texana* queen.

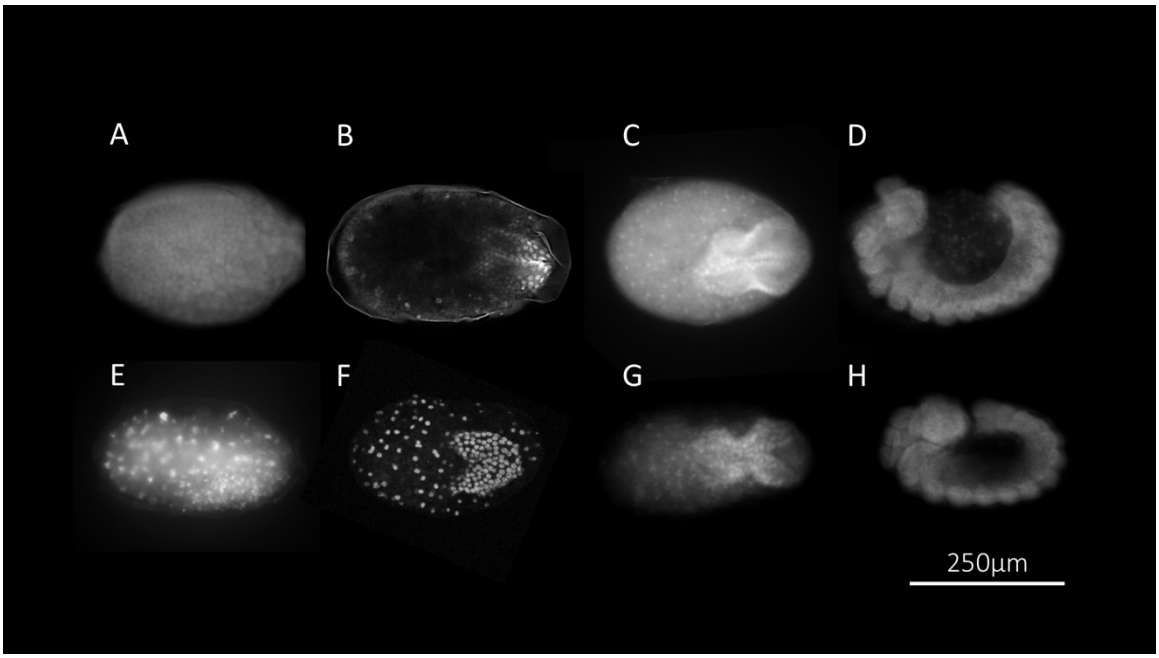

Figure 2.9. Embryogenesis comparison of *Atta texana* (A-D) and *Mycocepurus smithii* (E-H). Multiple nuclei emerged on the surface of the embryos during blastoderm stage (A, E). The heart-shaped germ-band formed toward to the posterior end of the embryos (B, F). The germ-band started to curve from the lateral side and extend towards to the posterior side during embryonic gastrulation and elongation (C, G). Body segments formed during segmentation (D, H).

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Chapter 3: Colony fitness and garden growth in the asexual fungus-growing ant *Mycocepurus smithii* (Attini, Formicidae)

INTRODUCTION

After the nuptial flight of ants, a mated foundress queen faces many threats (e.g., predation, competition and opportunistic infection) that endanger the success of colony foundation (Weber 1966; Oster and Wilson 1978; Porter and Tschinkel 1986; Hölldobler and Wilson 1977; Keller 1991; Marti et al. 2015). These threats are exacerbated during nest-founding in fungus-growing ants (Attini), because queens need to sustain a symbiotic fungal garden in addition to nourishing the first brood of workers (Wheeler 1907; Weber 1958; Mueller et al. 1998; Fernández-Marín et al. 2005; Marti et al. 2015). During the first two days of nest founding, an attine foundress expels from her infrabuccal pocket a pelleted inoculum of fungal hyphae, brought by the foundress from her natal nest, as starter inoculum of her incipient garden (Weber 1982; Mueller et al. 2001). Using nutrients stored in her body (in fat body and degenerating flight muscles) and, in some attine species, some plant material brought into the nest as substrate for fungiculture, the queen then carefully nurtures her incipient garden and raises her first cohort of workers (Weber 1958; Weber 1966; Mueller et al. 1998; Marti et al. 2015). Only after eclosion of the first cohort of workers does the queen cease to tend the garden, and the workers then assume responsibility for cultivation of the fungus that is essential for the success of the incipient colony. In fungus-growing ants, the biomass of fungus garden, queen fecundity (number of eggs laid), and the resulting number of workers are therefore key fitness components (Himler et al. 2009; Kellner et al. 2018) that determine colony fitness of fungus-growing ants.

In the fungus-growing ant *Mycocepurus smithii*, low fecundity and possibly asexual reproduction by *M. smithii* foundresses are two disadvantages that impact the

success of the typical monogynous nest founding (Fernández-Marín et al. 2005; Himler et al. 2009; Rabeling et al. 2009; Rabeling et al. 2011). A colony founded by a single queen transitions to a polygynous phase only after surviving this haplometrotic nest-founding stage characterized by limited egg-laying potential of the foundress queen. For example, of 74 new *M. smithii* colonies surveyed by Fernández-Marín et al. (2005), 85% these 74 were monogyne colonies and 15% were polygyne colonies with 2-4 foundresses. In a second sample of an additional 109 incipient colonies, each colony contained between 0-5 eggs, 0-4 larvae, 0-2 pupae, and 0-4 workers during this early phase of early colony establishment (Fernández-Marín et al. 2005). Colonies of *M. smithii* therefore exhibit relatively low growth rates (Fernández-Marín et al. 2003, 2004), compared to other attine ants such as *Atta* where a single foundress rears around 100 workers in the first three months.

In addition to low fecundity, a second potential disadvantage during colony foundation is the obligate asexuality (thelytoky) in most populations of *M. smithii*, where queens produce both daughter workers and new reproductive females (gynes) asexually (Fernández-Marín et al. 2005; Himler et al. 2009; Rabeling et al. 2009; Rabeling et al. 2011). Unlike typical ants with sexual reproduction, there exists therefore no within-nest genetic variation between workers within an asexually reproducing colony of *M. smithii* that is founded by a single queen, potentially reducing disease resistance of the colony (Hughes and Boomsma 2004) or precluding genetically-based behavioral specializations (Julian and Fewell 2004). Moreover, for those populations lacking sexual recombination, deleterious mutations may accumulate that entail long-term evolutionary disadvantages (Butlin 2002; Rice 2002). Rabeling et al. (2011) found both sexual and asexual *M. smithii* populations in Amazonian Brazil, whereas only asexual populations have been found so far elsewhere across the range of *M. smithii* from northern Argentina to northern Mexico

and across the Caribbean islands (Kempf 1963; Mackay et al. 2004; Fernández-Marín et al. 2005; Rabeling et al. 2011). Despite the potential disadvantages of asexual preproduction, asexually reproducing *M. smithii* have a wider geographic distribution than the sexual populations (Rabeling et al. 2011). The ecological and genetic factors that contribute to the success of asexual colonies throughout most of the range of *M. smithii* are unknown (Rabeling et al. 2011).

Mature *M. smithii* colonies are highly polygynous (Rabeling 2004; Fernández-Marín et al. 2005; Rabeling et al. 2009, 2011). In field colonies, for example, between one and 23 queens can occur in the same reproductive nest (Fernández-Marín et al. 2005), and laboratory colonies can accumulate dozens to hundreds of reproducing queens coexisting in the same colony (Fang and Mueller, unpublished observations). However, Fernández-Marín et al. (2004, 2005) observed that 85-88% of incipient colonies in Panamá are founded by a single queen, indicating that most multi-queen nests in *M. smithii* become secondarily polygynous after a single-queen nest-founding phase. This suggests the hypothesis that *M. smithii* optimize colony fitness by transitioning at a specific queen-to-worker ratio from a monogynous phase during nest-founding to a subsequent polygynous phase.

To further elucidate factors that optimize colony fitness and garden growth in *M. smithii*, we varied the queen-to-worker ratio in small experimental colonies set up with a standardized fungus-garden biomass. Our experiments aimed to test (1) whether an incipient colony with multiple queens (pleometrosis) is able to grow and sustain a larger biomass of fungus garden than a single-queen colony (haplometrosis); (2) whether there exists an optimal number of workers that an incipient colony can support with a given garden biomass; and (3) whether there exists a specific queen-to-worker ratio when colonies transition from monogyny to polygyny.

MATERIALS AND METHODS

Ant colonies

All colonies of *M. smithii* were collected in Central Panamá using methods described previously (Rabeling et al. 2009; Kellner et al. 2013, 2015, 2018). Colonies were brought to a lab at the University of Texas at Austin and maintained there in a temperature-controlled room (25 ± 1 °C). Each colony was kept with its fungus gardens in a 7.8*7.8*3.0 cm^3 plastic container with a bottom of hydrated plaster, and this nest chamber was connected to a dry foraging chamber by a transparent vinyl tube, as described by Sosa-Calvo et al. (2015). As gardening substrate, each colony was given in the foraging chamber a mixture of polenta and finely minced ground oats (3:1 ratio polenta-to-oats by volume). The plaster in the garden chamber was rehydrated weekly to generate a humid environment for the fungus in the garden chamber.

Preparation of brood-free fungus garden fragments

To generate brood-free garden for the below four experiments, we removed garden fragments of about 4 cm^3 along with about 300 workers, removed all female reproductives (winged gynes and dealate queens), then waited 60 days to allow any brood in these fragments to develop to eclosion. This waiting time is a superior method to generate brood-free garden compared to searching several rounds through a garden to remove all brood, because the garden is sensitive to a low humidity environment, and carefully searching for embedded eggs and larvae requires teasing apart the garden into small fragments harms the garden. Sixty days is a sufficient time to rear all brood because the total development from an egg to an adult is 60 days under 25 ± 1 °C (Fang and Mueller, unpublished observation).

Basic experimental protocol

We randomly selected queens and workers for experiments from colonies of the so-called A5 ant-clone used in previous experiments by Kellner et al. (2013, 2018). Because colony fitness of lower-attine ants such as *M. smithii* is dependent on fungal garden weight (Mehdiabadi et al. 2006; Kellner et al. 2018), we standardized the weights of initial gardens by giving each colony a standardized amount of fungus garden at the beginning of an experiment (either 0.1 gram or 0.45 gram, depending on the experiment described below). The total mass of each garden was weighed weekly for the duration of the experiment (13-31 weeks, depending on the experiment), weighing gardens blindly with respect to experimental treatment to minimize observer bias (Kardish et al. 2015). For weighing of gardens, we anesthetized the ants by filling the chamber briefly with carbon dioxide, then gently removed all ants from each garden with soft forceps or moistened paintbrushes, then weighed the garden on a weighing dish, then returned all ants to their garden chamber.

We generated experimental nests with different combinations of number of queens and number of workers to test for the effect of queen-to-worker ratio on garden growth:

Experiment 1: Effect of the number of mature queens per colony

According to the field survey by Fernández-Marín et al. (2005), founding colonies of *M. smithii* contain zero to four foundresses (average of 1.16 foundresses). To explore the effect of foundress queen number on colony growth and garden growth, we set up 26 experimental colonies with different number of queens, either no queen (6 replicates), one queen (7 replicates), five queens (5 replicates), or ten queens (8 replicates). Each colony was given 30 workers and 0.1 gram of garden (prepared as described above).

Because workers of *M. smithii* continue to maintain gardens even in the absence of a queen, it was possible to conduct this experiment with queenless colonies as a control. Starting in January 2017, all colonies were observed and weighted (see above) for 31 weeks.

Experiment 2: Effect of the number of workers per colony

In September 2017, we set up experimental colonies each with 0.1 gram of garden and one queen, but varied worker numbers, either 30 workers (N = 9 colonies), 60 workers (N = 3 colonies), or 90 workers (N = 2 colonies). Each colony was observed for 13 weeks. In January 2018, we added seven more replicate colonies to each treatment and observed these for 13 weeks. Combining the samples from 2017 and 2018, the total experimental colonies with 30, 60, 90 workers were 16, 10, and 9, respectively.

Experiment 3: Effect of the number of workers per colony with different initial fungus weight

To further determine the effects of worker number and a garden larger than the one used in Experiment 2, we set up colonies with single queens and with either 6, 18, 60, or 90 workers, and provided each colony with and initial garden size of 0.45 gram (N = 3 for each treatment). Starting in April 2018, each colony was monitored for 13 weeks as described above.

Experiment 4: Testing cues for production of new queens

In Experiment 2, new daughter queens (gynes) were produced in some colonies by the fourth month (January 2018) from three colonies each with 60 workers and one queen, and from one colony with 90 workers and one queen. These colonies had reached by then fungus-garden weights averaging about 0.45 gram. To test the roles of worker

number and garden size, we set up new colonies each with one queen and either 60 or 90 workers, and gave each of those 0.45 gram of initial fungus garden (N = 3 for each of these two treatments). Starting in June 2018, each colony was monitored for 13 weeks as described above. We combined this dataset with the dataset from Experiment 2 and compared garden weights from colonies with 60 workers (N = 10 colonies, 3 colonies from 2017 and 7 from 2018) and 90 workers (N = 9 colonies, 2 colonies from 2017 and 7 from 2018).

Experiment 5: Quantification of egg-laying capacity of single queens

To estimate the number of eggs laid by a single queen of *M. smithii*, we randomly selected 313 queens of *M. smithii* from source colonies of the most productive ant clone (the so-called A5-clone; Kellner et al. 2013) and set up three replicate experimental runs (run A with N = 98 individual queens; run B with N = 104 queens; run C with N = 111 queens) (Fig. 3.6). Each queen was set up individually in a Petri dish (6 cm diameter) with a bottom of 1% agarose to maintain humidity and allow easy visual identification of any eggs laid by the queen on the agarose. We counted the number of eggs laid by each queen after 24 hours.

Statistical analysis

For Experiments 1 and 3, we used a Poisson Generalized Linear Mixed Model (GLMM) with worker number as the fixed effect and time (week) as the random effect (Bolker et al. 2009). For Experiment 2, we combined the small dataset from 2017 with a dataset (seven additional replicates) from 2018, and analyzed this combined dataset with the same GLMM also used in Experiments 1 and 3, but with two random effects, week and year. For Experiment 4, as explained above, we also combined the dataset from

Experiment 2 (10 and 9 replicates for 60 and 90 workers provided with 0.1 gram of garden) with the dataset from June 2018 (3 and 3 replicates for 60 and 90 workers provided with 0.45 gram of garden), and analyzed this combined dataset with the same linear mixed model but with two random effects, week and year. We performed statistical analyses in RStudio, version 1.1.463 (R Core Team 2017), and generated plots using ggplot2. We include R scripts (Table C1) in the Appendix C, statistics for pairwise comparisons (Tables 3.1-3.6), and the raw data .csv files on GitHub website (Tables S1a-6a; https://github.com/ChiChunAndyFang/AntSurvival). For Experiment 5, we analyzed the data with analysis of variance (ANOVA) and a Tukey's post-hoc test for identifying differences among the three replicate runs.

RESULTS

We use the following notation to abbreviate experimental treatments that vary the number of workers and the number of queens: 0Q-30W denotes an experimental setup of no queen and 30 workers; 5Q-30W denotes 5 queens and 30 workers, and likewise for other queen-to-worker ratios.

Experiment 1: Effect of the number of mature queens per colony

We found no significant difference in fungus garden weight between colonies in the 0Q-30W (control) and 1Q-30W treatments ($P = 0.5885$, Fig. 3.1). The significant differences were found between 0Q-30W colonies with both 5Q-30W colonies ($P < 0.01$; Fig. 3.1; Table 3.1) and 10Q-30W colonies ($P < 0.01$; Fig. 3.1; Table 3.1). The presence of multiple queens (5Q-30W and 10Q-30W) significantly reduced colonies fitness compared to the 1Q-30W colonies ($P < 0.01$; Fig. 3.1; Table 3.1). The estimated intercept was 0.1668, and the estimated coefficient for 1Q-30W colonies, 5Q-30W colonies, and

10Q-30W colonies were 0.0061, -0.0473, and -0.0926, respectively. By week 31, only one replicate each survived for 0Q-30W colonies and for 1Q-30W colonies; all the replicates of 5Q-30W colonies and 10Q-30W colonies had died by week 19 and 27, respectively. To analyze how colony fitness was influenced by the different numbers of queen(s) at the founding colony, we used a GLMM to analyze the first 13 weeks of the experiment. We only compared treatment 1Q-30W, 5Q-30W, and 10Q-30W treatments, which most closely resemble the queen-to-worker ratios observed in field colonies (Fernández-Marín et al. 2003). The GLMM analyses show that 1Q-30W colonies had significantly greater garden weights than 10Q-30W colonies ($P < 0.01$; Fig. 3.2), and 5Q-30W colonies had significantly greater garden weights than 10Q-30W colonies ($P = 0.01$; Fig. 3.2). All pairwise comparisons between treatments are listed in Table 3.2.

Experiment 2: Effect of the number of workers per colony

Because Experiment 1 indicated that founding colonies with single queens had the highest fitness (Figs. 3.1 & 3.2), we used in Experiment 2 single-queen colonies but varied worker number from 30, 60, and 90 workers, giving each colony 0.1 gram fungus garden when starting the experiment in September 2017. We generated 9, 3, and 2 replicate colonies, respectively, for the 1Q-30W, 1Q-60W, and 1Q-90W treatments. Because the small sample size of colony 1Q-90W ($N = 2$) seemed insufficient, we increased samples sizes by starting in January 2018 seven additional replicate colonies for each treatment. Combining the replicates from 2017 and 2018, the total sample sizes were therefore $N = 16$, 10, and 9 for 1Q-30W, 1Q-60W, and1Q-90W treatments, respectively. In an analysis using a linear mixed model that treated both week and year as the random effects in the combined dataset, 1Q-60W colonies had significantly greater garden weights than 1Q-30W colonies ($P < 0.01$; Fig. 3.3), and 1Q-30W colonies had

significantly greater garden weights than 1Q-90W colonies ($P < 0.01$; Fig. 3.3). When analyzing only the 2018 data with a linear mixed model with only one random effect (i.e., week), 1Q-60W colonies had significantly greater garden weights than 1Q-30W colonies ($P < 0.01$, Fig. 3.7). There was no significant difference in garden weights between 1Q-30W colonies and 1Q-90W colonies ($P = 0.4984$, Fig. 3.7; Table 3.6). All pairwise comparisons between Experiment 2 treatments are listed in Table 3.6.

Among the 14 experimental colonies ($N = 9, 3, 2$ for 1Q-30W, 1Q-60W, and 1Q-90W, respectively) started in September 2017, new daughter queens (winged females, i.e., gynes) were first observed in the fourth month (January 2018) in three 1Q-60W colonies and in one of the 1Q-90W colonies. The first new daughter queen appeared in the eighth month (May 2018) in two colonies of the 1Q-30W treatment. In the replicate colonies added in 2018 ($N = 7$ for 1Q-30W, 1Q-60W, and 1Q-90W), no new daughter queens appeared from the 1Q-60W or 1Q-90W colonies by the fourth month (May 2018) of the experiment.

Experiment 3: Effect of the number of workers per colony with different initial fungus weight

There was no significant difference in garden weights between 1Q-6W and 1Q-18W colonies ($P = 0.409$; Fig. 3.4). 1Q-60W and 1Q-90W colonies had significantly greater garden weights than 1Q-18W colonies ($P < 0.05$; Fig. 3.4). All pairwise comparisons between treatments are listed in Table 3.4.

Experiment 4: Testing cues for production of new queens

In Experiment 2, new daughter queen appeared during the fourth month (January 2018) in three 1Q-60W colonies, and in one of the 1Q-90W colonies. The average garden weight in these four colonies was around 0.45 gram. We therefore used in Experiment 4

colonies with either 60 or 90 workers along with 1 queen, but increasing the initial the garden weight to 0.45 gram garden (N = 3 for each of these two treatments). The experiment was started in June 2018, and each colony was observed for 13 weeks. There was no significant difference in garden weight between 1Q-60W colonies with 0.1 gram fungus garden and 1Q-90W colonies with 0.1 gram fungus garden (P = 0.579; Fig. 3.5). Both 1Q-60W and 1Q-90W colonies with initially 0.45 gram fungus had greater garden weights than 1Q-60W colonies with initially 0.1 gram fungus (P < 0.01; Fig. 3.5). All pairwise comparisons between treatments are listed in Table 3.5. We observed no new daughter queens during the 4 months of this experiment.

Experiment 5: Quantification of egg-laying capacity of single queens

On average, each queen laid 1.12 ± 0.06 eggs within 24 hours. There were no significant differences in eggs laid among the three replicate runs (P = 0.2168). A Tukey's post hoc-test also showed no significant difference when comparing each pair of replicate run (df = 2; A-B: P = 0.998; A-C: P = 0.307; B-C: P = 0.273). When combining three replicate runs, the percentage for 0, 1, 2, 3, 4, 5 eggs laid in 24 hours were 33.86%, 35.14%, 19.16%, 8.62%, 2.55%, 0.63%, respectively (Fig. 3.6). About 90% of the queens therefore laid 2 or less eggs in 24 hours.

DISCUSSION

Our experiments aimed to compare colony fitness in nests founded by either multiple queens (pleometrosis) or single queens (haplometrosis) in the asexual, polygynous fungus-growing ant *Mycocepurus smithii*. Colony fitness was greater for haplometrotic than pleometrotic colonies over the course of a 31-week experiment (Fig. 3.1), and the presence of supernumary queens under pleometrosis appears to have

stressed a limited resource, the initial fungal garden, during the early stages of colony establishment. Specifically, increased brood in colonies with supernumary queens, in addition to the queens themselves, may have led to overconsumption of garden, diminished garden growth, and thus resource shortage. Consequently, during the first 13 weeks of colony establishment in Experiment 1 (Fig. 3.2), haplometrotic colonies produced larger gardens, a proxy of fitness. The greater fitness of haplometrotic compared to pleometrotic colonies observed in our experiments may also be true for natural field conditions, where most nests are founded by single queens (85% haplometrosis, 15% pleometrosis; Fernández-Marín et al. 2005). In contrast, in the leafcutter ant *Acromyrmex versicolor*, incipient colonies with single foundresses are less likely to establish a successful fungus garden than colonies with cooperating co-foundresses (Cahan and Julian 1999). This pattern is consistent with observations from other social insects where pleometrosis can enable cooperating queens to conserve energy reserves, which translates into increased brood production, faster colony growth, more efficient foraging behavior, and higher colony survival (Tchinkel and Howard 1983; Adams and Tchinkel 1995; Johnson 2004; Overson et al. 2014; Chiu et al. 2018).

Transition from monogyny to polygyny

Although haplometrosis is the typical and apparently also optimal strategy during the nest-founding phase of *M. smithii* (Figs. 3.1 & 3.2), mature *M. smithii* colonies are highly polygynous (Rabeling 2004; Fernández-Marín et al. 2005; Rabeling et al. 2009, 2011). Fernández-Marín et al. (2005) therefore hypothesized that *M. smithii* in the field become secondarily polygynous after a monogyne nest-founding stage. This colony ontogeny must involve a crucial transition point where the addition of supernumary queens increases growth of the colony and the fungus garden, unlike the early nest-

founding stage were supernumery queens reduce garden growth (Figs. 3.1 & 3.2). That is, to support supernumerary queens, the colony needs to possess sufficient fungus garden, cultivated and maintained by a sufficient number of workers. In our second experiment, the optimal switch point from a single-queen to a multiple-queen phase is when the colony reaches a ratio of about 1 queen to 60 workers (1Q-60W), a ratio at which colonies can sustain the largest biomass of fungus garden when initially given 0.1 gram of fungus garden (Fig. 3.3 & 3.7). Our third experiment confirmed that queen-to-worker ratios of 1Q-60W and 1Q-90W maximize garden growth when colonies were started with, respectively, 0.45 gram of fungus garden (Fig. 3.4).

An optimal queen-to-worker ratio is therefore critical for both incipient and mature colonies of *M. smithii*: an inadequate number of workers for a given size of garden results in deficient garden maintenance, whereas an excess number of supernumerary queens reduces garden growth because the food demands by the queens and their brood exceeds the carrying capacity of a garden. Our findings are consistent with the previous studies that initial workers number contributes critically to colony survival and competitive ability in both monogynous and polygynous ant species (Bartz and Hölldobler 1982; Rissing and Pollock 1991; Tschinkel and Howard 1983; Hölldobler and Wilson 1990).

Production of new queens

In our experiments, new daughter queens (gynes) were found only in colonies with specific queen-to-worker ratios. For example, in the fourth month of Experiment 2, new daughter queens (gynes) were produced by colonies with 1Q-60W and 1Q-90W that started with 0.1 gram of fungus garden. In contrast, colonies with 1Q-30W and 0.1gram garden needed twice the time (8 months) to produce the first daughter queens, and also by

the fourth month, no gynes appeared in colonies with the same queen-worker combinations that started initially with 0.45 gram of fungus in Experiment 4 (Fig. 3.5). We therefore hypothesize that new daughter queens are added in *M. smithii* under specific queen-to-worker ratios of ants tending a specific size of fungus garden. Caste-ratio-dependent production of new queens has also been observed in monogynous colonies of *Acromyrmex*, where new sexuals are produced when fungus gardens reach a specific threshold size (Bekkevold and Boomsma 2000). Our finding is the first such detailed study for *M. smithii* documenting the complete stepwise transitions from monogyny to polygyny through the production of supernumary queens.

Limits on egg-laying capacity and colony growth rate in *Mycocepurus smithii*

For eusocial insects, fitness is maximized by optimizing the colony output of female and male reproductives and the quality of these reproductives (Ratnieks and Reeve 1992). In the asexual *M. smithii*, overall colony output of female reproductives is limited in part by the low fecundity of queens, which, under optimal laboratory conditions (25 ± 1 °C; well-fed mature colonies), can produce only 1.12 ± 0.06 (SE) eggs per day (Fig. 3.6). Each queen of *M. smithii* has one pair of ovaries, each ovary is composed of three ovarioles, and each of the total of six ovarioles can generate only a single egg per day. This limits the number of eggs that a single queen can lay per day to maximally six eggs, but most queens exhibit a much lower daily fecundity (Fig. 3.6). In our experiment quantifying egg-laying potential: 69% of the queens (216 of a total of 313 queens tested) laid one or no egg within 24 hours in our experiment quantifying egg-laying potential (Fig. 3.6). Because the queens used in this experiment were selected from the most productive ant clone A5 in our laboratory, this low fecundity suggests that typical field colonies may exhibit even lower productivity than in our experiment.

A second reason for the low growth rate of *M. smithii* colonies is the relatively long developmental time from egg to adult. *M. smithii* eggs take around 20 days from oviposition to hatching, and developmental from first instar larva to a worker adult lasts around 40 days at 25°C (Fang et al., in preparation). Because gynes are slightly larger than workers, total development requires 60-70 days for a gyne at 25°C in the laboratory (Fang and Mueller, unpublished observations). This slow development time is similar to the one observed for *M. smithii* in the field in Panamá (Fernández-Marín et al. 2005) where the first workers emerge 2-5 months after colony founding. This suggests the hypothesis that the mother queen produces new gynes to overcome the slow growth rate by adding supernumary queen that in *M.smithii* are clonally identical to the mother queen (Fernández-Marín et al. 2005; Himler et al. 2009; Rabeling and Kronauer 2013), and these supernumary queens therefore contribute to brood production and consequently faster colony growth. This model of life-history strategy and optimal colony growth likely applies to other clonal ant species as well (Rabeling and Kronauer 2013).

Implications for caste determination

When and how queen/worker caste is determined in ants – at an early developmental stage or late larval instar stage – is still unclear (Wheeler 1986; Grbic et al. 1997; Khila and Abouheif 2008, 2010). Some evidence suggest that caste could be determined during the earliest embryological stages involving gene-regulation that channels development into a worker or queen path already at the egg stage (Khila and Abouheif 2008, 2010). Other evidence suggests that caste can be influenced late in larval development, for example in those ant species where environmental factors and a nest's social environment affect caste determination in brood (Hölldobler and Wilson, 1990; Wheeler 1994). Likewise, nutrients can influence caste determination (Richards and

Packer 1994; Wheeler 1994), but also the queen-to-worker ratio and other such factors that depend on colony size (Boulay et al. 2009; Schmit et al. 2011; Ruel et al. 2012). In addition, cues from eggs, larvae, and workers can influence caste determination in brood (Boulay et al. 2007; Endler et al. 2004; Warner et al. 2016). Our experiments on *M. smithii* add more evidence that caste determination is influenced by factors in the nest environment. Specifically, *M. smithii* gynes were produced in our experiments only after the colony cultivated sufficient fungus garden weight, which was dependent on an optimal queen-to-worker ratio for maximal garden growth. While these findings document the importance of environmental factors in the caste determination of *M. smithii*, this does not bear on the controversy regarding the timing of early versus late caste determination in brood development (Wheeler 1986; Grbic et al. 1997; Khila and Abouheif 2010). Documenting the importance of environmental factors does not bear on this controversy because it is possible that either (a) queens of *M. smithii* prime caste-development early by laying queen-destined eggs when a fungus garden reaches a sufficient size, or alternatively and independent of any queen factors, (b) the nest environment directly influences brood development at either egg, early larval, or late larval stage. Because of the ease of manipulating queen-to-worker ratios and garden size in *M. smithii*, in addition to easy propagation in the laboratory because of its clonal reproduction, *M. smithii* could be a model system to elucidate the above processes of either queen-mediated or environmental caste determination.

Shortcomings of our study and recommendations for future research

Our study has several shortcomings, which do not invalidate the above findings, but which can be addressed in future investigations of *M. smithii*. First, we used CO_2 to anesthesize ants for weekly weighing of colonies; this procedure may have reduced the

productivity of *M. smithii* queens, as exposure of honey bee queens to CO_2 reduces ovary activity (Berger et al. 2015; Koywiwattrakul et al. 2005). However, although we used CO_2 treatment because it simplifies the weighing procedure, all experimental replicates were treated the same, so the observed differences between experimental treatments cannot be attributed to CO_2 exposure per se, but to other parameters varied in the experiments (e.g., queen-to-worker ratios). Second, because we selected wingless *M. smithii* queens randomly from our laboratory colonies, and not winged foundress queens that may disperse from their natal nest under natural conditions, it is possible that the behavior of some queens in our experiments differed from those of true foundress queens. Founding queens observed in nature (semi-claustral queens) exhibit behaviors that distinguish them from queens under laboratory conditions (Fernández-Marín et al. 2004). In the field, semi-claustral foundress queens of *M. smithii* forage near their incipient colony for garden substrate rather than staying always in the safety of their nest (Fernández-Marín et al. 2004), whereas mature queens presumably do not forage in established nests with workers. This difference in queen behavior between our laboratory colonies and natural colonies may limit the extent to which our findings can be extrapolated to wild *M. smithii* foundresses. Third, fungus garden growth exhibited an inverted U curve in all our experiments (Figs. 3.1, 3.3, 3.4, 3.5 & 3.7), where garden weights increased initially for a period of the first 4-6 weeks, then garden weights declined until the end of each experiment. We do not know the exact reason for the eventual decline in garden weights in our experiments, but we suspect that a gradually increasing pathogen load may cause the reduction of fungus garden. Although the plastic containers used to house the experimental colonies were sterilized at the start of each experiment, pathogens (e.g., mites, pathogenic fungi or bacteria) may accumulate during the multi-month experiments and eventually impede garden growth. Pathogens infect

incipient attine colonies also in the field, as queens that found nests of social insects generally experience high mortality rates (Marti et al. 2015), but laboratory colonies may be plagued by higher pathogen loads than those found under natural conditions in the safety below ground. An improved experimental design therefore could regularly switch experimental colonies to new, sterilized containers (e.g., every 2-3 months). In fact, such a colony maintenance regime switching colonies regularly to new containers has allowed us to keep the same colony-clones of *M. smithii* (e.g., the A5 clone) for nearly 20 years in the laboratory.

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TABLES

Table 3.1. Pairwise comparisons of garden weights over 31 weeks between all treatments in Experiment 1, analyzed using emmeans::emmeans() in R. The *P*-values are adjusted using a Tukey method for comparing differences between 4 treatments (*M. smithii* treatments 0Q-30W, 1Q-30W, 5Q-30W, and 10Q-30W). Figure 3.1 presents the corresponding data graphically.

contrast	estimate	SE	df	t.ratio	p.value
0Q30W - 1Q30W	-0.00612	0.0113	772	-0.541	0.9489
0Q30W - 5Q30W	0.04731	0.0123	772	3.844	0.0008
0Q30W - 10Q30W	0.09262	0.011	772	8.438	<.0001
1Q30W - 5Q30W	0.05343	0.0119	772	4.49	<.0001
1Q30W - 10Q30W	-0.09874	0.0105	772	-9.387	<.0001
5Q30W - 10Q30W	-0.04531	0.0116	772	-3.911	0.0006

Table 3.2. Pairwise comparisons of garden weights over 13 weeks between all treatments in Experiment 1, analyzed using emmeans::emmeans() in R. The *P*-values are adjusted using a Tukey method for comparing differences between 3 treatments (*M. smithii* treatments 1Q-30W, 5Q-30W and 10Q-30W). Figure 3.2 presents the corresponding data graphically.

contrast	estimate	SE	df	t.ratio	p.value
1Q30W - 5Q30W	0.0616	0.0215	245	2.862	0.0126
5Q30W - 10Q30W	-0.0532	0.0209	245	-2.542	0.0312
1Q30W - 10Q30W	-0.1148	0.019	245	-6.038	<.0001

Table 3.3. Pairwise comparisons of garden weights over 13 weeks between all treatments in Experiment 2, analyzed using emmeans::emmeans() in R. The *P*-values are adjusted using a Tukey method for comparing differences between 3 treatments (*M. smithii* treatments 1Q-30W, 1Q-60W and 1Q-90W). Week and year (2017 and 2018) were treated as the random effects. Figure 3.3 presents the corresponding data graphically.

contrast	estimate	SE	df	t.ratio	p.value
1Q30W - 1Q60W	-0.066	0.0176	440	-3.744	0.0006
1Q30W - 1Q90W	-0.0518	0.0181	440	-2.852	0.0126
1Q60W - 1Q90W	0.0142	0.0181	438	0.785	0.7124

Table 3.4. Pairwise comparisons of garden weights over 13 weeks between all treatments in Experiment 3, analyzed using emmeans::emmeans() in R. The *P*-values are adjusted using a Tukey method for comparing differences between 4 treatments (*M. smithii* treatments 1Q-6W, 1Q-18W, 1Q-60W and 1Q-90W). Figure 3.4 presents the corresponding data graphically.

contrast	estimate	SE	df	t.ratio	p.value
1Q6W - 1Q18W	0.0276	0.0334	140	0.828	0.841
1Q6W - 1Q60W	0.2157	0.0334	140	6.464	<.0001
1Q6W - 1Q90W	-0.3024	0.0334	140	-9.061	<.0001
1Q18W - 1Q60W	-0.1881	0.0334	140	-5.635	<.0001
1Q18W - 1Q90W	-0.2747	0.0334	140	-8.232	<.0001
1Q60W - 1Q90W	-0.0867	0.0334	140	-2.597	0.0504

Table 3.5. Pairwise comparisons of garden weights over 13 weeks between all treatments in Experiment 4, analyzed using emmeans::emmeans() in R. The *P*-values are adjusted using a Tukey method for comparing differences between 4 treatments (*M. smithii* treatments 1Q-60W + 0.1 gram fungus, 1Q-90W + 0.1 gram fungus, 1Q-60W + 0.45 gram fungus and 1Q-90W + 0.45 gram fungus). Week and year (2017 and 2018) were treated as the random effects. Figure 3.5 presents the corresponding data graphically.

contrast	estimate	SE	df	t.ratio	p.value
1Q60W_0.1 - 1Q60W_0.45	-0.2627	0.0307	308	-8.56	<.0001
1Q60W_0.1 - 1Q90W_0.1	0.0116	0.0209	308	0.555	0.945
1Q60W_0.1 - 1Q90W_0.45	-0.3494	0.0307	308	-11.384	<.0001
1Q60W_0.45 - 1Q90W_0.1	0.2743	0.0307	308	8.941	<.0001
1Q60W_0.45 - 1Q90W_0.45	-0.0867	0.037	308	-2.342	0.0909
1Q90W_0.1 - 1Q90W_0.45	-0.361	0.0307	308	-11.766	<.0001

Table 3.6. Pairwise comparisons of garden weights over 13 weeks between all treatments in Experiment 2, analyzed using emmeans::emmeans() in R. The *P*-values are adjusted using a Tukey method for comparing differences between 3 treatments (*M. smithii* treatments 1Q-30W, 1Q-60W and 1Q-90W). Week was treated as the random effect. Figure 3.7 presents the corresponding data graphically.

contrast	estimate	SE	df	t.ratio	p.value
1Q30W - 1Q60W	-0.07537	0.0142	258	-5.29	<.0001
1Q30W - 1Q90W	0.00966	0.0142	258	0.678	0.7766
1Q60W - 1Q90W	0.08503	0.0142	258	5.968	<.0001

FIGURES

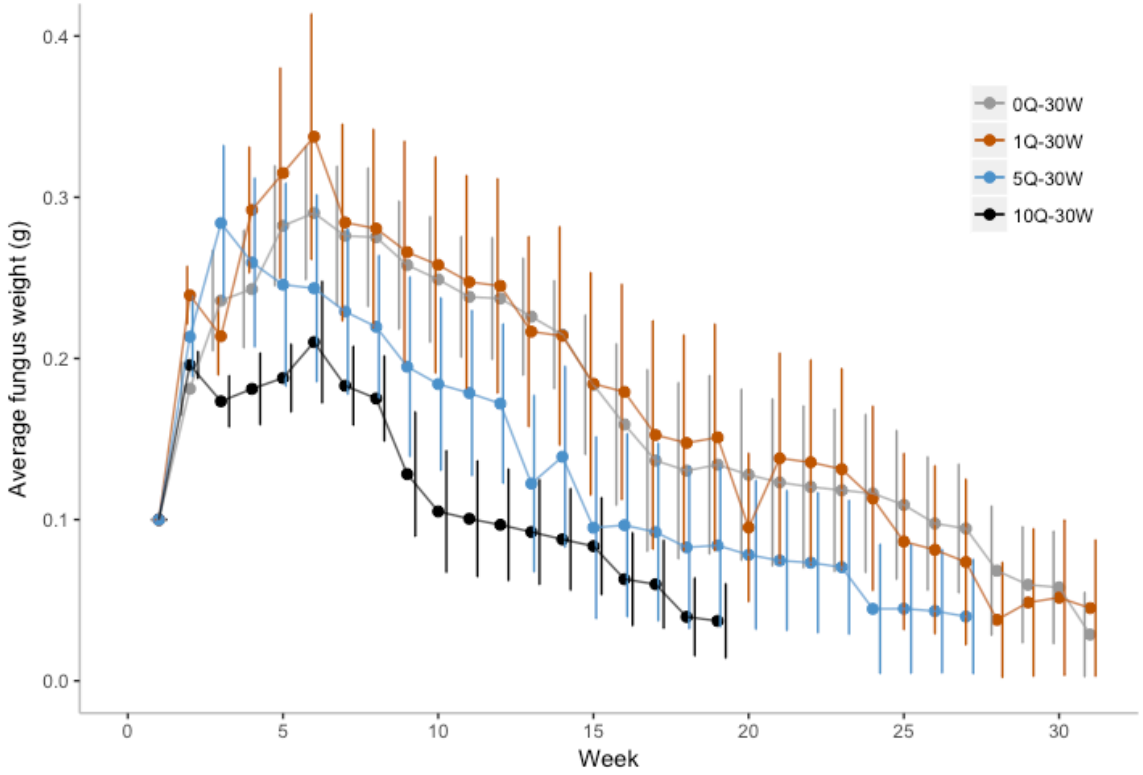

Figure 3.1. Weekly fungus garden weight (in gram; mean ± SEM) over 31 weeks for *Mycocepurus smithii* nests starting with different number of queens (0, 1, 5, or 10 queens) and 30 workers. Overall, there was no significantly difference between 0Q-30W colonies (control, gray) and 1Q-30W colonies (burnt orange) ($P = 0.5885$). The estimated intercept was 0.1668, and the estimated coefficient for 1Q-30W colonies (gray), 5Q-30W colonies (blue), and 10Q-30W colonies (black) were 0.0061, -0.0473, and -0.0926, respectively. Negative estimated coefficients indicate a decrease of fungus garden weight over time. The colonies with either 5 queens or 10 queens had significantly lower garden weights than the garden weights of 0Q-30W control colonies (both $P < 0.01$). Garden weights of 1Q-30W colonies were significantly greater than garden weights of 5Q-30W colonies ($P < 0.01$, Table 3.1). Treatment differences were analyzed using a linear mixed model which treated queen number as the fixed effect, and week as the random effect. For 0Q-30W and 1Q-30W colonies, there was only one replicate alive in week 31. All the replicate colonies in the 5Q-30W and 10Q-30W treatments had died by week 19 and 27, respectively. Sample sizes were $N = 6$, 7, 5, and 8 for treatments 0Q-30W, 1Q-30W, 5Q-30W, and 10Q-30W, respectively. Q = number of queens. W = number of workers.

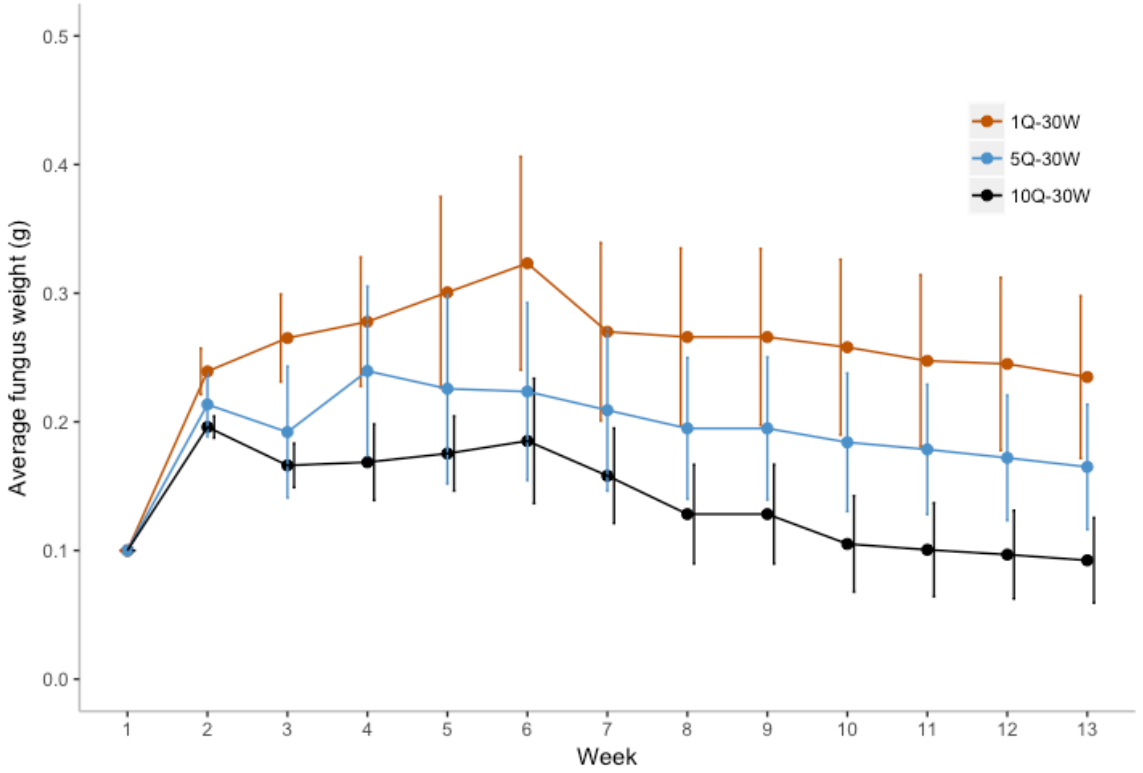

Figure 3.2. Weekly fungus garden weight (in gram; mean ± SEM) over 13 weeks for *Mycocepurus smithii* nests starting with different number of queens (1, 5, or 10 queens), 30 workers, and an initial garden of 0.1 gram. The 1Q-30W colonies had significantly greater garden weights than 10Q-30W colonies ($P < 0.01$). The 5Q-30W colonies had significantly greater garden weights than 10Q-30W colonies ($P = 0.01$). Treatment differences were analyzed using a linear mixed model where queen number was treated as the fixed effect and week was treated as the random effect. Sample sizes were $N = 7$, 5, and 8 for treatments 1Q-30W, 5Q-30W, and 10Q-30W, respectively. Q = number of queens. W = number of workers.

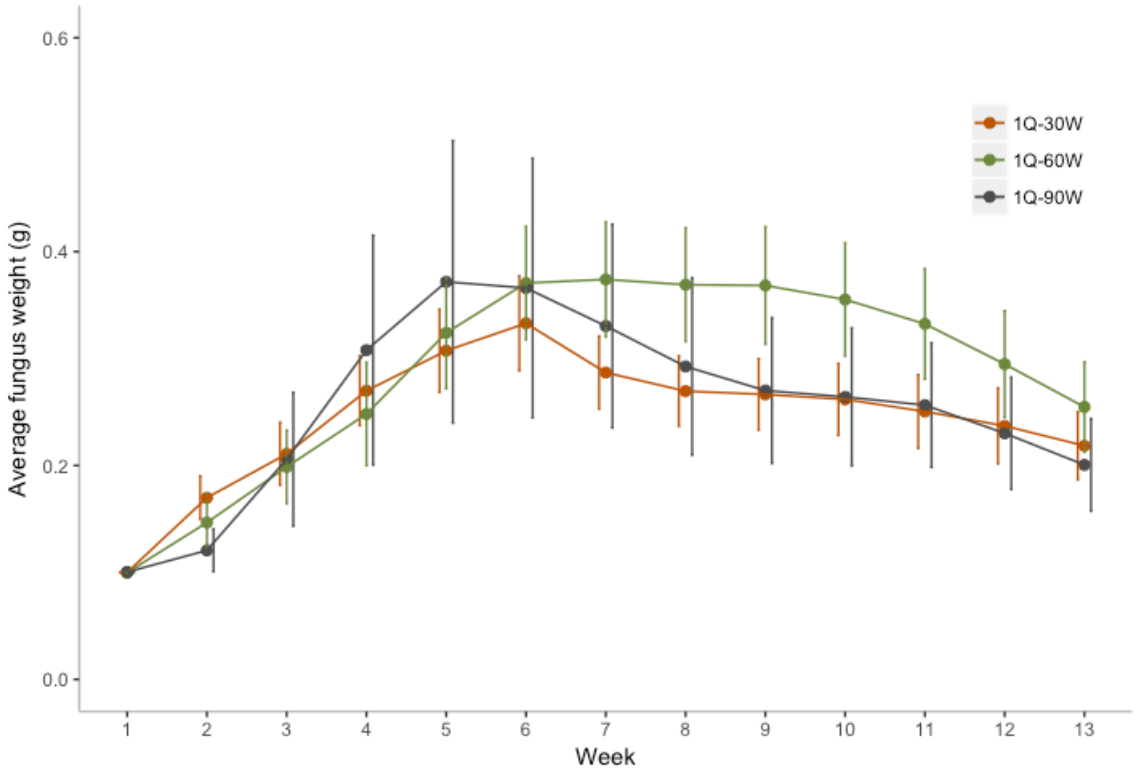

Figure 3.3. Weekly fungus garden weight (in gram; mean ± SEM) over 13 weeks for Mycocepurus smithii nests starting with different number of workers (30, 60, or 90 workers), 1 queen per colony, and an initial garden of 0.1 gram. The data combine experimental replicates from 2017 (14 replicates) and 2018 (21 replicates). The 1Q-60W colonies had significantly greater garden weights than 1Q-30W colonies ($P < 0.01$). In addition, there is a significant difference in garden weights between 1Q-30W colonies and 1Q-90W colonies ($P < 0.01$). Treatment differences were analyzed using a linear mixed model where queen number was treated as the fixed effect; week and year (2017 and 2018) were treated as the random effects. Sample sizes were $N = 16$, 10, and 9 for treatments 1Q-30W, 1Q-60W, and 1Q-90W, respectively. Q = number of queens; W = number of workers.

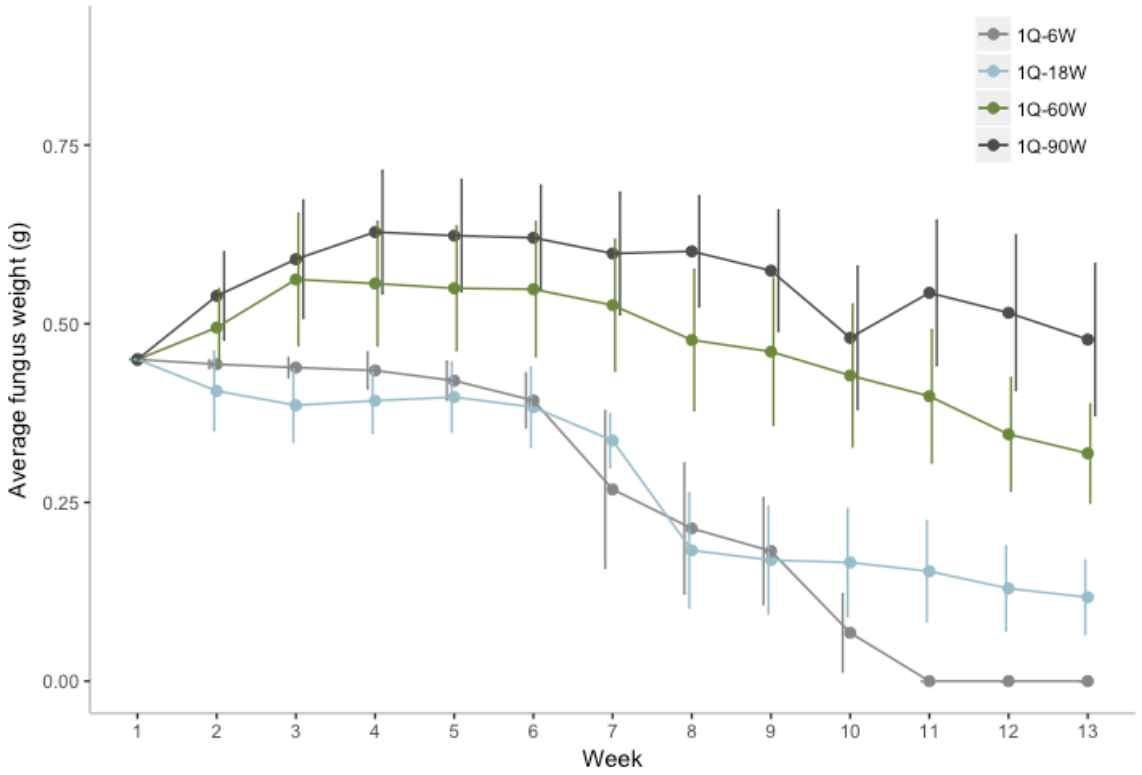

Figure 3.4. Weekly fungus garden weight (in gram; mean ± SEM) over 13 weeks for *Mycocepurus smithii* nests starting with different number of workers (6, 18, 60, or 90 workers), 1 queen per colony, and an initial garden of 0.45 gram. There is no significant difference in garden weights between 1Q-6W colonies and 1Q-18W colonies ($P = 0.409$). Garden weights of both 1Q-60W and 1Q-90W colonies were significantly greater than garden weights of 1Q-18W colonies ($P < 0.05$). There is no significant difference in garden weights between colonies with 1Q-60W and colonies with 1Q-90W ($P = 0.0504$, Table 3.4). Treatment differences were analyzed using a linear mixed model where worker number was treated as the fixed effect and week was treated as the random effect. Sample sizes were $N = 3$ for each of the four treatments 1Q-6W, 1Q-18W, 1Q-60W, and 1Q-90W. Q = number of queens. W = number of workers.

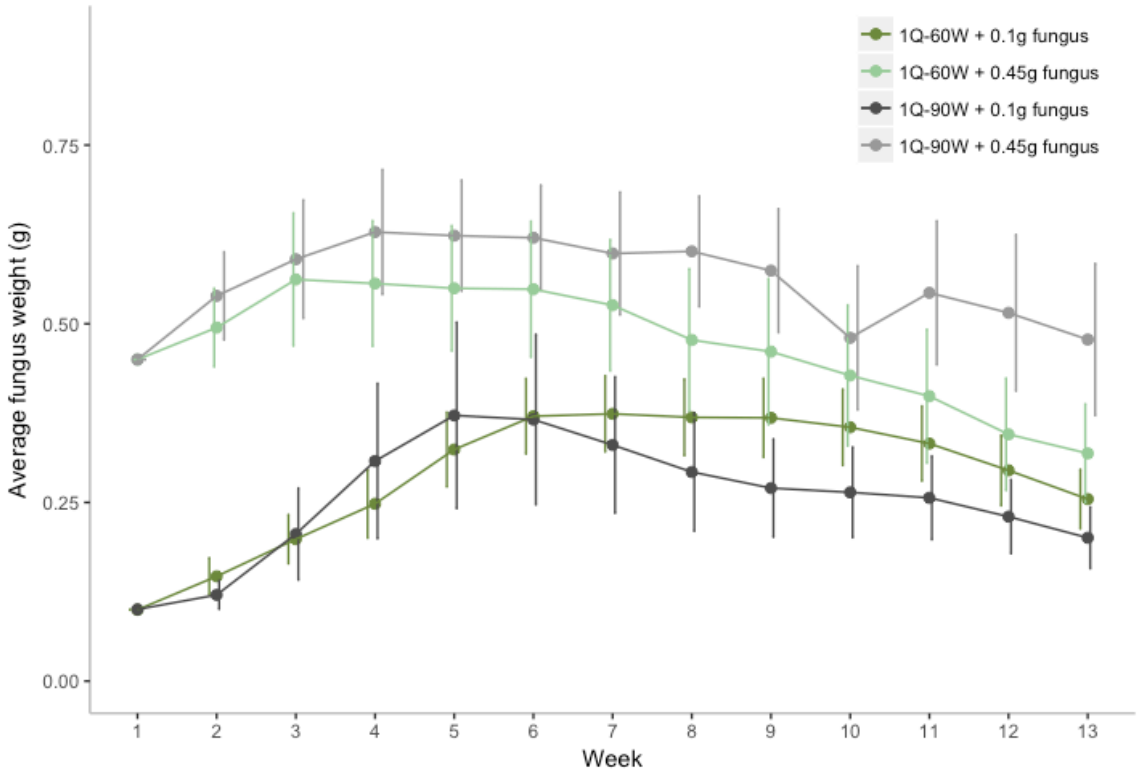

Figure 3.5. Weekly fungus garden weight (in gram; mean ± SEM) over 13 weeks for *Mycocepurus smithii* nests starting with different number of workers (60 or 90 workers), 1 queen per colony, and initial garden weights of either 0.1 or 0.45 gram. There is no significant difference in garden weights between colonies with 1Q-60W + 0.1 gram fungus and colonies with 1Q-90W + 0.1 gram fungus ($P = 0.579$). In addition, there is no significant difference in garden weights between colonies with 1Q-60W + 0.45 gram fungus and colonies with 1Q-90W + 0.45 gram fungus ($P = 0.0909$, Table 3.5). Not surprising, both colonies starting with 1Q-60W + 0.45 gram fungus and colonies with 1Q-90W + 0.45 gram fungus had significantly greater garden weights than colonies starting with 1Q-60W + 0.1 gram fungus ($P < 0.01$). Treatment differences were analyzed using a linear mixed model that worker number was treated as the fixed effect, and both week and year were treated as the random effects. Sample sizes were $N = 10$, 9, 3, and 3 for treatments 1Q-60W + 0.1 g fungus, 1Q-90W + 0.1 g fungus, 1Q-60W + 0.45 g fungus, and 1Q-90W + 0.45 g fungus, respectively. Q = number of queens. W = number of workers.

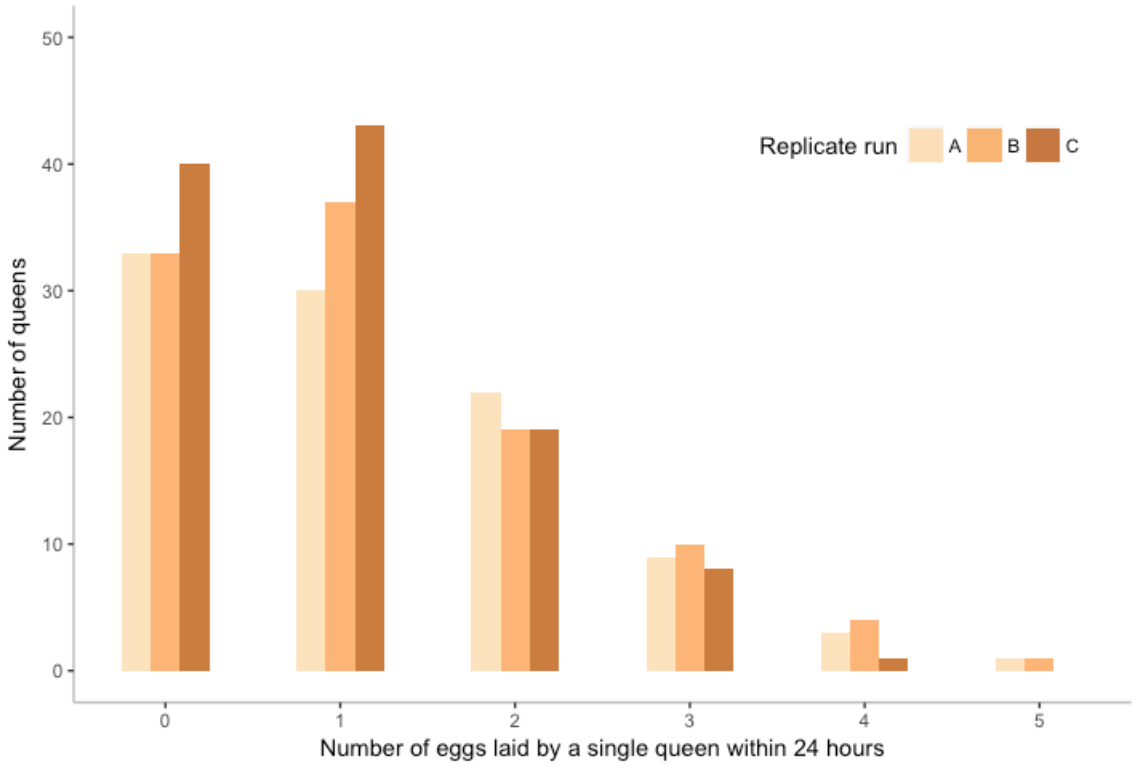

Figure 3.6. Queen egg-laying productivity within 24 hours in *Mycocepurus smithii*. Each queen was randomly selected from the fungus garden of a large source colony. We placed each queen in a separate container and counted the number of eggs laid with 24 hours. The experiment was replicated in three separate runs, and egg-laying rates did not differ in any comparison between any two replicate runs (ANOVA, $df = 2$, $P = 0.217$). There was also no significant difference between any two replicate runs in the number of eggs laid using a Tukey's post hoc test (A-B: $P = 0.9987$; A-C: $P = 0.3076$; B-C: $P = 0.2731$). The number of the queens tested in replicate run A, B, and C were respectively 98, 104, and 111. On average, each queen laid 1.12 ± 0.06 (SE) eggs within 24 hours.

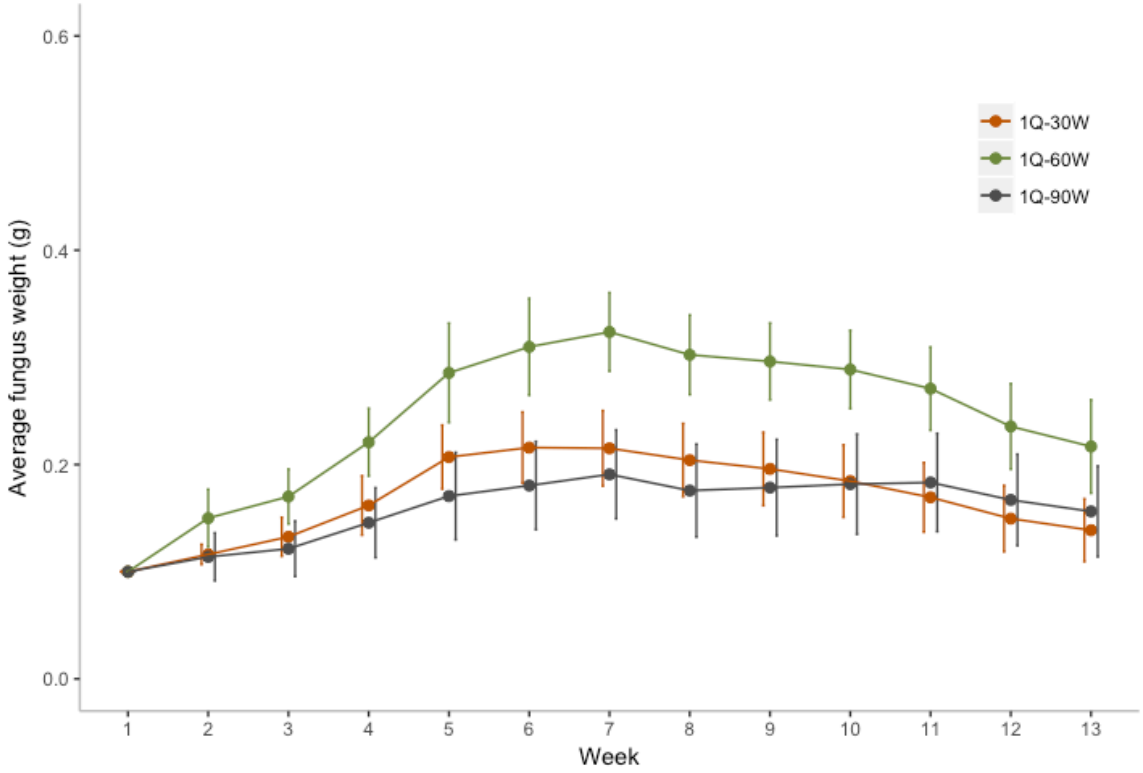

Figure 3.7. Weekly fungus garden weight (in gram; mean ± SEM) over 13 weeks for *Mycocepurus smithii* nests starting with different number of workers (30, 60, or 90 workers), 1 queen per colony, and an initial garden of 0.1 gram. The experimental series here includes only the replicate colonies from 2018 (seven replicates for each of the three treatments). The 1Q-60W colonies had significantly greater garden weights than 1Q-30W colonies ($P < 0.01$). There was no significant difference in garden weights between 1Q-30W colonies and 1Q-90W colonies ($P = 0.4984$). There is no significant difference in garden weights between colonies with 1Q-30W and colonies with 1Q-90W ($P = 0.7766$, Table 3.6). Treatment differences were analyzed using a linear mixed model where queen number was treated as the fixed effect and week was treated as the random effect. Sample sizes were $N = 7$, 7, and 7 for treatments 1Q-30W, 1Q-60W, and 1Q-90W, respectively. Q = number of queens; W = number of workers.

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Chapter 4: A new type of egg produced by foundress queens of *Atta texana* (Attini, Formicidae)

INTRODUCTION

Fungus-growing ants (attines) are unique among ants because they depend on a mutualistic fungus that they grow as food (Mehidabadi and Schultz 2009). The fungus is vertically transmitted between ant generations, as a female reproductive takes an inoculum of the fungus from her natal nest, stores a pellet of fungus for the duration of the mating flight in a pocket in her mouth, then uses this pellet as a starter culture for her first fungus-garden in the incipient nest (Huber 1905, 1907; Autuori 1942; Bazire-Bénazet 1957; Weber 1972; Mueller et al. 2001; Mueller 2002; Augustin et al. 2011). Most attine foundresses forage occasionally at this early stage for plant substrate to sustain the growth of the incipient garden (Wheeler 1907; Weber 1958; Mueller et al. 1998; Fernández-Marín et al. 2005), but *Atta* foundresses have claustral nest founding where they seal themselves into their incipient nest, precluding foraging. An *Atta* foundress therefore has to sustain the growth of the incipient garden entirely on the resources carried by the foundress (von Ihering 1898; Huber 1905, 1907; Autuori 1942; Weber 1972; Seal 2009; Augustin et al. 2011). Only after the queen has raised her first cohort of workers does a colony forage for leaf material to sustain fungal growth. To nourish the incipient garden and the first brood, a foundress queens lays two types of eggs, reproductive eggs that develop into workers and trophic eggs that the queen processes to feed to her brood, or that the queen ingests herself to digest and provide to the fungus in the form of the queen's feces (Huber 1905, 1907).

Trophic eggs are non-embryonated eggs incapable of development, and they serve as a supplementary nutrient for queens and larvae (Huber 1905, 1907; Autuori 1942; Wilson 1971; Weber 1972; Glancey et al. 1973; Diehl-Fleig and de Araújo 1996;

Augustin et al. 2011; Lee et al. 2017; Peeters 2017). In ants, the size of trophic eggs varies markedly between different species, and trophic eggs can either be larger or smaller than reproductive eggs within a species (Glancey et al. 1973; Augustin et al. 2011; Lee et al. 2017). In some ant species, workers lay trophic eggs to help feed the larvae (Gobin et al. 1998; Lee et al. 2017; Peeters 2017), in other species trophic eggs are produced only by foundress queens during the early nest founding stage to nourish the first cohort of workers (Weber 1972; Glancey et al. 1973). In incipient colonies of *Atta*, for example, larvae are fed only trophic eggs laid by the newly-mated foundresses (Huber 1905, 1907; Autuori 1942; Bazire-Bénazet 1957; Weber 1972), presumably because consumption of fungus garden by the brood would attenuate garden growth. Trophic eggs represent the most important food resources for both foundresses and larvae at the nest-founding stage of *Atta sexdens* (Bazire-Bénazet 1957; Augustin et al. 2011), and *At. colombica* foundress can rear brood to eclosion even in the absence of fungal garden (Fernández-Marín and Wcislo 2005), indicating that trophic eggs are a sufficient food for complete development of *Atta* larvae.

During experiments to characterize embryological development in *Atta texana* (Fang et al. in preparation; Chapter 2 in this dissertation), we discovered a third kind of egg laid by foundress queens in addition to reproductive and trophic eggs. We (a) identify this third type of egg with fluorescent microscopy as undeveloped reproductive eggs; (b) observed that these undeveloped reproductive eggs are eventually digested by the fungus, whereas reproductive eggs are not digested and thus complete embryonic development; (c) show that trophic eggs liquify by some endogenous process within 24 hours after oviposition; and (d) found one incipient nest that produced nanitic males (possibly diploid males) that were killed by this foundress shortly after eclosion.

MATERIALS AND METHODS

Ant colonies

Dealate foundresses of *Atta texana* were collected on 5. May 2018 at Brackenridge Field Laboratory (N = 25 females) and on 21. May 2018 at Commons Ford Metropolitan Park (N = 30 females), both in Austin, Texas, USA. Foundresses were collected as they were excavating nests during the day following mating flights at dawn (Marti et al. 2015). Each foundress was kept in a round plastic dish (5.5cm diameter; 3.7cm height), as described in detail by Marti et al. (2015). Each dish had a bottom layer of 2.5 cm moistened plaster, leaving enough room above the plaster to approximate the natural chamber dimensions dug by *At. texana* foundresses. The plaster was saturated with distilled water to maintain 100% humidity in the chamber. Prior to the experiment, the layer of plaster in each dish had been exposed to UV light for 10 minutes to reduce contamination. All lab colonies were maintained in a temperature-controlled room (25 ± 1 °C) during the experiments. Foundresses were not given any substrate for gardening, and foundresses therefore used their stored body resources for oviposition, brood rearing, and gardening, as is typical for natural claustral colony-founding of *At. texana* and other *Atta* species (Della Lucia et al. 1995; Fujihara et al. 2012; Seal 2009; Marti et al. 2015).

Colony maintenance and egg collection

Atta foundresses begin oviposition and expel a pellet of fungal hyphae during the first two days of nest founding (Autuori 1942; Weber 1972; Augustin et al. 2011; Marti et al. 2015). Because of the high mortality typical for incipient colonies (typically more than 50%; Hölldobler and Wilson 1990, 2010; Peeters and Ito 2001; Brown and Bonhoeffer 2003; Marti et al., 2015; Camargo et al. 2016), we waited with egg collection until one week after the first brood of workers emerged. The first workers eclosed about 45 days

after the day of the mating flight (at 25 ± 1 °C). Of the 55 foundresses, 20 foundresses succeeded at cultivating a healthy fungus garden by 28. June 2018; however, only six of these colonies (all collected on 5. May) produced workers. Experimental eggs were collected from these 6 colonies every three days from 28. June to 16. August. Because eggs can be difficult to locate in gardens, we separated each queen for egg collection from her garden and placed her for 24 hours in a separate round plastic dish (5.5cm diameter; 3.7cm height) together with 3 of her minima workers and a small fragment from her garden (~0.5*0.5*0.2 cm^3). Each of these dishes had a bottom-layer of 1% agarose and was maintained at 25 °C ± 1 °C. The agarose maintained 100% humidity in a dish, but allowed also easy visual identification of any eggs on the smooth, translucent agarose substratum. Workers move any newly-laid eggs to the small fungus fragment, from which the eggs could then be collected with the help of a moistened fine brush. Because the main focus of our study was to characterize reproductive and trophic eggs, we did not collect behavioral observations on queens and workers systematically, but recorded observations sporadically while monitoring experimental dishes during the experiments.

Egg-deliquescence experiments

We observed reproductive eggs (R-egg), undeveloped reproductive eggs (UR-egg), and tropic eggs (T-egg) during egg collection (see details below; Figs. 4.1 & 4.2). Only the trophic T-eggs have been known so far as a source of nutrients for brood, the queen, and the garden in *Atta* (Huber 1905, 1907; Autuori 1942; Bazire-Bénazet 1957; Weber 1972; Augustin et al. 2011), but both UR-egg and T-egg could be a potential resource of nutrients for developing brood, the queen, or the garden. Preliminary observations indicated that the T-egg deliquesced within 24 hours after oviposition.

Specifically, these T-eggs liquified when the eggshell disintegrated, typically within a day, while R-eggs and UR-eggs did not deliquesce during that time. To quantify the deliquescence process more rigorously, and to characterize differences among the three types of eggs, we set up three simple experiments to test factors that may cause the T-eggs to liquify. First, the fungus may secrete factors, such as enzymes, that digest and liquify the T-eggs; this hypothesis predicts that contact with fungal garden is necessary, and that the T-eggs will not liquify (or liquify at a slower rate) when isolated from contact with fungal garden. Second, T-eggs may liquify by some unknown endogenous process, such as self-digestion of the thin eggshell; this hypothesis predicts that contact with fungal garden or with other eggs is not necessary for liquification to proceed. Third, R-eggs or UR-eggs may secrete factors that cause liquification of T-eggs; this hypothesis predicts that T-eggs will liquify when in contact with other eggs, but not when isolated without any contact in garden and when resting on an inert surface.

To test the predictions of these hypotheses, we set up simple experiments to observe whether liquification of T-eggs occurred under different conditions. To test whether contact with fungus was sufficient, we conducted parallel experiments where newly collected T-eggs were placed either onto a small piece of garden (~0.2*0.2*0.2 cm^3), or alternatively on an inert surface. For these experiments, eggs in isolation or eggs on garden were conducted in the absence of workers by placing them onto a central island (Fig. 4.3) in a sealed dish with a moat of distilled water surrounding the island to maintain humidity. This test chamber was a 6 cm diameter Petri dish with a central island of 1% agarose and a microscopy coverslip (Fig. 4.3) onto which we transferred with the help of a moistened fine brush newly-laid T-eggs (4-6 hours since oviposition) onto a garden fragment, then sealed the dish with Parafilm to maintain 100% humidity, then observed the liquification process for the next 24 hours. We repeated this experiment

with 20 T-eggs placed without contacting other eggs onto a garden fragment (Fig. 4.4). In a parallel experiment testing whether contact with fungal garden is necessary, we set up T-eggs on a neutral surface in the test chamber with 100% humidity. We repeated this experiment 10 times. To test whether contact between eggs was necessary, we added T-eggs and R-eggs to garden-fragments in test chambers such that T-eggs were in contact with R-eggs or UR-eggs. We repeated this experiment with 20 T-eggs placed in contact with other eggs onto a garden fragment (Fig. 4.5). We scored whether T-eggs had liquified after 24 hours, and we photographed representative results to illustrate the deliquescence process (Figs. 4.3-4.5).

Microscopy

To collect eggs for fluorescent microscopy, we moved a foundress into a small round container (Pioneer Plastics, Inc.; 6cm diameter, 4cm height) with a bottom of 1% agarose to provide humidity, a small garden fragment (~3 mm diameter), and 5 minima workers to move eggs laid by the queen into the small garden. We allowed the queen to lay eggs for 24 hours, then returned the queen back to her colony, but permitted the workers to tend to the newly-laid egg in the agarose chamber for some time to collect eggs of a known age (detailed methods in Fang et al in preparation and Chapter 2 of this dissertation). Using standard methods of embryo fixation (Fang et al. in preparation), we fixed 5-day old reproductive eggs (R-eggs) and undeveloped reproductive eggs (UR-eggs) to compare any embryo development between the two types of eggs. The embryo fixation and DAPI staining protocol are as same Fang et al. (in preparation, Chapter 2 in this dissertation). Fluorescent-microscopy images were taken with a Zeiss Axiovert Fluorescent Light Microscope at the Microscopy and Imaging Facility at the University of Texas at Austin (http://sites.cns.utexas.edu/cbrs/microscopy).

We measured the length and width of reproductive eggs (R-egg) and undeveloped reproductive eggs (UR-egg) using a Nikon Eclipse Ni Compound Light Microscope. Due to the fragility of tropic eggs (T-egg) that easily burst when transferred onto a microslide, we imaged T-eggs on the agarose substratum onto which they had been placed by the queen, using a Leica MZ16 Stereomicroscope fitted with a DFC420 digital camera, then calibrated each egg's length and width with ImageJ (https://imagej.nih.gov/ij/). Sample sizes for measurements of the three types of eggs are listed in Table 4.1.

RESULTS AND DISCUSSION

Comparison of the three types of eggs produced by *Atta texana* queens

Trophic eggs (T-eggs) are larger than the other two eggs, and undeveloped reproductive eggs (UR- eggs) are slightly larger than reproductive eggs (R-eggs) (Figs. 4.1i & 4.2). The raw data of egg length measurement .csv file is uploaded to the GitHub website (Table S7; https://github.com/ChiChunAndyFang/TrophicEggMeasurement). The average length (mean ± SD) of R-eggs, UR-eggs, and T-eggs was 426.09 ± 16.98 μm, 491.04 ± 35.53 μm, and 640.54 ± 80.01 μm, respectively (Table 4.1). The average width of R-egg, UR-egg, and T-egg was 239.75 ± 11.67 μm, 317.96 ± 17.33 μm, and 530 ± 70.82 μm, respectively (Table 4.1). The average length of the three types of eggs were significantly different from each other (Tukey's post-hoc test, $P < 0.01$, Fig. 4.2), suggesting that workers may be able to recognize the three types of eggs by their size differences. The length of reproductive eggs of *At. texana* (≈425μm length) are similar to those of other leaf-cutting ants studied so far. For example, we deduced from published figures that the length of reproductive eggs is ≈420μm long in *At. cephalotes* and ≈440μm in *At. sexdens* (Dijkstra et al. 2005); ≈470 μm in *Acromyrmex rugosus rugosus* (Verza et al. 2017); and ≈460-620μm in *Ac. echinatior* and ≈460-625μm in *Ac.*

octospinosus (for presumably pooled samples of reproductive and trophic eggs; Dijkstra et al. 2005).

T-eggs deliquesced (i.e., liquified) within 24 hours after being laid by the queen (Fig. 4.1g & 4.1h). Previous studies had reported the morphology of T-eggs from *At. sexdens* (Augustin et al. 2011), but the process of egg-deliquescence is fully described here for the first time (Fig. 4.1g & 4.1h, Figs. 4.3-4.5). Because of the similar size between R-eggs and UR-eggs, it appears that UR-eggs have been overlooked in previous studies of oviposition behavior in *Atta*, but fluorescent microscopy documents that UR-eggs are clearly distinct from R-eggs (Fig. 4.6). R-eggs produced by a fertilized *At. texana* foundresses undergo embryogenesis and develop for about 15 days at the egg stage (at 25 ± 1 °C; see Chapter 2 in this dissertation) before hatching, but UR-eggs do not develop and do not hatch. For example, when comparing R-eggs with UR-eggs that are both 5 days old (Fig. 4.6), DAPI staining revealed that germ band elongation is complete by day 5 in R-eggs, showing a high density of nuclei where the future head lobe is located (see arrow in Fig. 4.6a). In contrast, 5-day-old UR-eggs show a single nucleus (see arrow in Fig. 4.6b), indicating that no embryo developed in UR-eggs. UR-eggs therefore fail to develop properly, and it is likely that UR-eggs are eventually consumed by the ants or digested by the fungus (Fig. 4.1d-f). Because UR-eggs comprised about 10% of all the eggs that we collected for our experiments, it is possible that UR-eggs are a maladaptive artifact produced by *At. texana* under the unusual laboratory conditions. Alternatively, UR-eggs may represent a second kind of trophic egg that does not liquify within 24 hours (see further observations below), whereas T-eggs evolved as a readily liquifying trophic egg. Future studies on *At. texana* and other fungus-growing ant species may be able to evaluate any adaptive function of UR-eggs.

Fate of the three types of eggs in incipient gardens

In our observations of incipient colonies, minima workers gathered the R-eggs from the queen, placed them on the incipient garden, and tended to the eggs with continuous grooming and guarding (Fig. 4.1a). Some R-eggs appeared to be neglected by the workers occasionally. Although these neglected R-eggs can be covered by live mycelium, the eggs maintained their integrity without being damaged or digested by the fungus (Fig. 4.1b & 4.1c). In contrast, putative UR-eggs were digested by mycelium by the eighth day after oviposition (Fig. 4.1d). The nutrients of UR-egg were presumably absorbed by the mutualistic fungus (Fig. 4.1e & 4.1f), suggesting recycling of nutrients from defunct eggs by the fungus, or perhaps, as discussed above, that UR-eggs may possibly represent a second type of trophic egg. T-egg invariably deform morphologically (Fig. 4.1g) when placed on a hard surface (see arrow in Fig. 4.2), indicating that trophic eggs to not have a rigid eggshell maintaining the shape of the egg, therefore readily liquify and can therefore be readily imbibed by the ants or digested by the fungus. T-egg eggshells are clearly more fragile than the eggshells of the rigidly chorionated R-eggs and UR-egg, and additional studies are needed to fully characterize eggshell properties.

Egg-deliquescence experiments

Trophic eggs (T-eggs) deliquesce even by themselves within 24 hours (Fig. 4.3b & 4.3c, $N = 10$) when placed on a solid surface in a humid experimental chamber (Fig. 4.3a), indicating that T-eggs are sufficiently sturdy to withstand oviposition and the initial transport by the ants, but that T-eggs become quickly fragile by some endogenous process, and eventually liquify (Fig. 4.3b & 4.3c) to facilitate imbibing by the ants or digestion by the fungus. T-eggs placed experimentally on a fungus garden fragment liquified within 24 hours, regardless of whether these T-eggs were placed in isolation

(Fig. 4.4, $N = 20$) or placed in contact with other eggs onto a garden fragment (Fig. 4.5, $N = 20$). Contact between eggs is therefore not necessary for deliquescence of T-eggs in garden. These experiments establish the first complete timeline of trophic-egg deliquescence in *Atta*, an important adaptation for nourishing not only the broods but also the incipient fungus garden.

Foundress behavior

Our study was not designed to collect systematic observations of foundress behavior in *At. texana*, but we sporadically observed two interesting behaviors of foundresses towards T-eggs. First, an *At. texana* foundress uses her mandibles as a scoop to gently scoop up a trophic egg (T-egg) from her abdomen during oviposition, such that a T-egg comes to rest on the closed mandibles functioning as a scoop, whereas a foundress picks up the smaller R-eggs and UR-eggs by holding them with her mandibles functioning as pincers. This behavioral difference indicated that the foundress recognizes the fragility of the T-eggs, and the scooping behavior appears to minimize the chance of bursting the egg during transport. Interestingly, workers carried T-eggs by using the mandibles as pincers, possibly because the mandibles of minima workers may be too small to function as an effective scoop. As a second behavioral observation, we observed one foundress that produced only nanitic males during the colony founding stage, but no workers (Fig. 4.7). This *At. texana* foundress had been collected after the mating flight on 5. May 2018, and we found two nanitic male adults and one nanitic male pupa on 19. July 2018 in this queen's garden. The nanitic males may have been diploid males that were homozygous at the sex-determining locus, most likely resulting from inbreeding (Ross and Fletcher 1985b; Duchateau and Marien 1995; Gerloff et al. 2003), but other explanations cannot be ruled out without further study, such as developmental mutations

in a diploid female, or unusual development of diploid eggs under the accelerated embryogenesis and larval ontogeny typical for brood of foundress queens (see Chapter 3 in this dissertation). Diploid males were found in the field in some mature colonies of *Atta sexdens* from Panamá, and these diploid males are somewhat larger than normal haploid males of *At. sexdens* (Armitage et al. 2010). If the nanitic males of *At. texana* can be shown to be diploid, their ontogeny would therefore have to be different from those of the larger diploid males of *At. sexdens*. In our incipient nest of *At. texana*, the foundress groomed the male pupa before that male's eclosion (Fig. 4.7a), but the same queen killed and then dismembered the two nanitic males within 48 hours after the males' eclosion (Fig. 4.7d). Because foundress queens appear to kill nanitic males quickly, it is possible that such males may have been overlooked in previous studies of incipient *Atta* nests.

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TABLES

Type	Length	Width	*N*
R-egg	426.09 ± 16.98	239.75 ± 11.67	44
UR-egg	491.04 ± 35.53	317.96 ± 17.33	49
T-egg	640.54 ± 80.01	530.00 ± 70.82	28

Table 4.1. Average length and width of the three types of eggs (in µm, ± SD) laid by *Atta texana* foundresses: reproductive eggs (R-eggs), undeveloped reproductive eggs (UR-eggs), and trophic eggs (T-eggs). T-eggs do not have a rigid eggshell and easily deform when resting on a surface (Fig. 4.2 & 4.3a), which may have contributed to the greater variances of T-egg measurements compared to the measurements of R-eggs and UR-eggs.

FIGURES

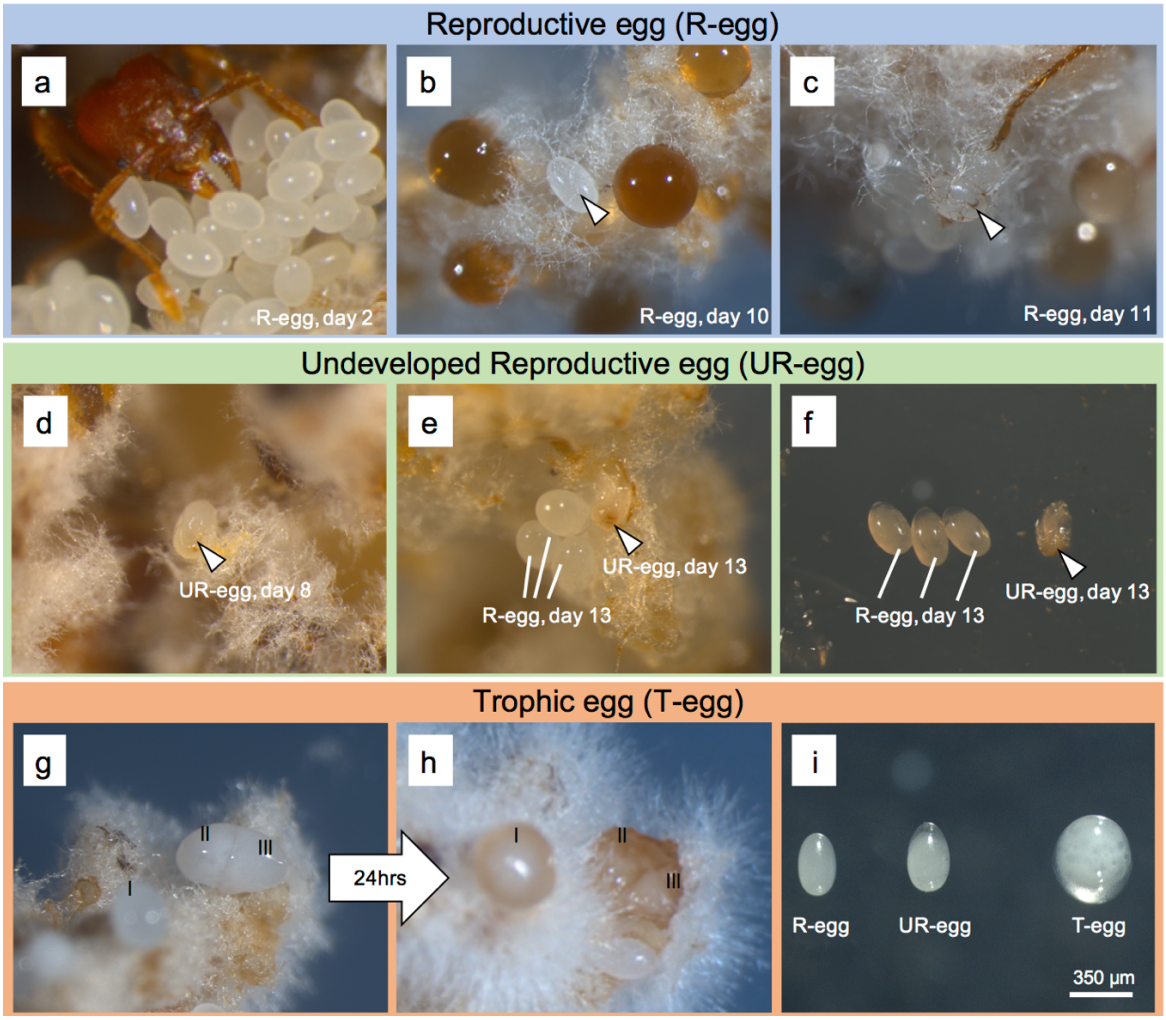

Figure 4.1. Comparative morphology of three types of eggs laid by *Atta texana* foundresses: reproductive eggs (R-eggs), undeveloped reproductive eggs (UR-eggs), and trophic eggs (T-eggs). a. A cluster of R-eggs tended by an *At. texana* worker. b. A 10-day old R-egg (see arrow) with healthy mycelium growing on the egg surface. c. An 11-day old R-egg with dead or stressed mycelium on the egg surface (see arrow). d. An UR-egg starting to be digested by the mycelium, causing a depression on the surface (see arrow). e. & f. An UR-egg was digested by the mycelium by day 13 (see arrow). g & h. Three T-eggs (labeled I, II, and III) all laid on the same day, then all three eggs deliquesced within 24 hours. i. Morphological aspects of a R-egg, UR-egg, and T-egg, showing that T-eggs are larger than the other two types of eggs, and that UR-eggs are slightly larger than R-eggs (see Table 4.1 for exact size measurements).

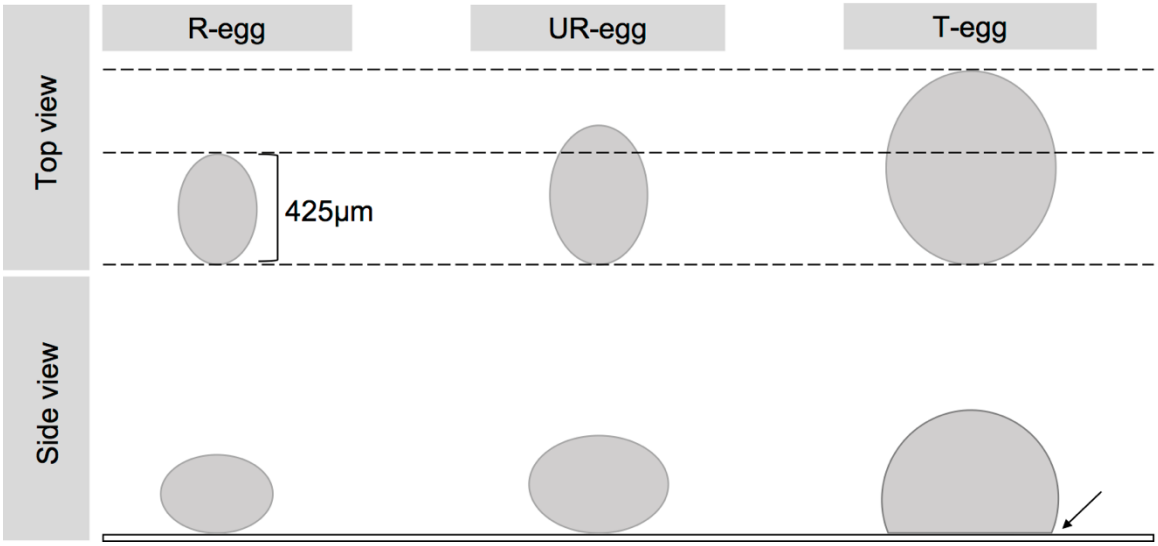

Figure 4.2. Size comparison of a reproductive egg (R-egg), an undeveloped reproductive egg (UR-egg), and a trophic egg (T-egg) laid by an *Atta texana* foundress. The top view shows the increase in length between a reproductive egg (average of 425µm), undeveloped reproductive egg (490µm), and trophic egg (640µm). The side view shows the morphological deformation at the bottom (see arrow) in a trophic egg (T-egg) when resting on a solid surface, whereas R-eggs and UR-eggs are firm and do not deform when resting on a surface.

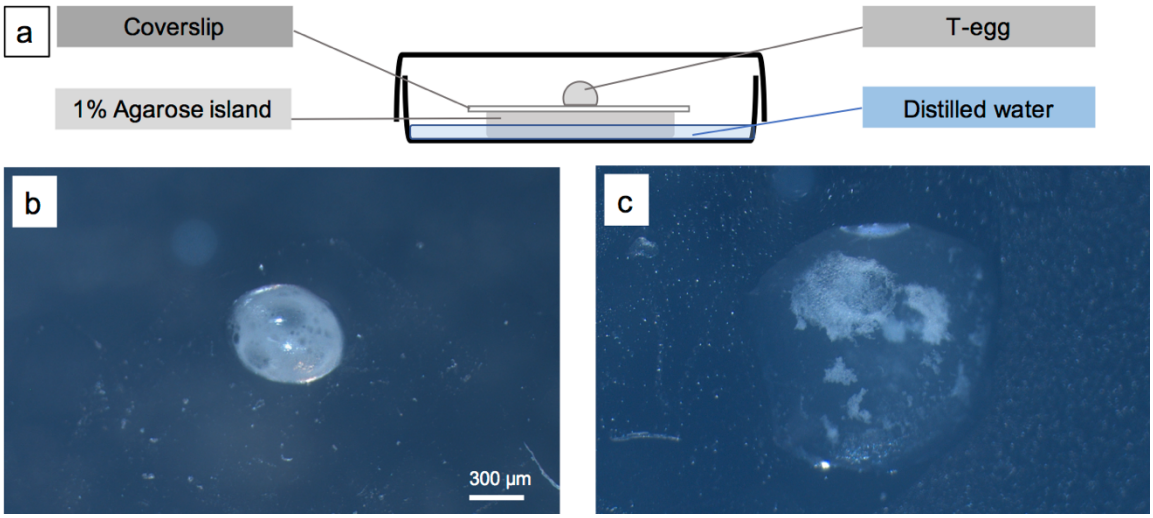

Figure 4.3. a. Experimental setup testing whether trophic eggs (T-eggs) liquify by themselves as a result of endogenous processes. b. At the beginning of the experiment, one newly-laid T-egg was placed on the island shown in a. and resting on a cover slip and maintained 25 ± 1°C. To maintain the humidity, distilled water was added into the Petri dish, then the dish is sealed with parafilm. c. A T-egg that had liquified within 24 hours.

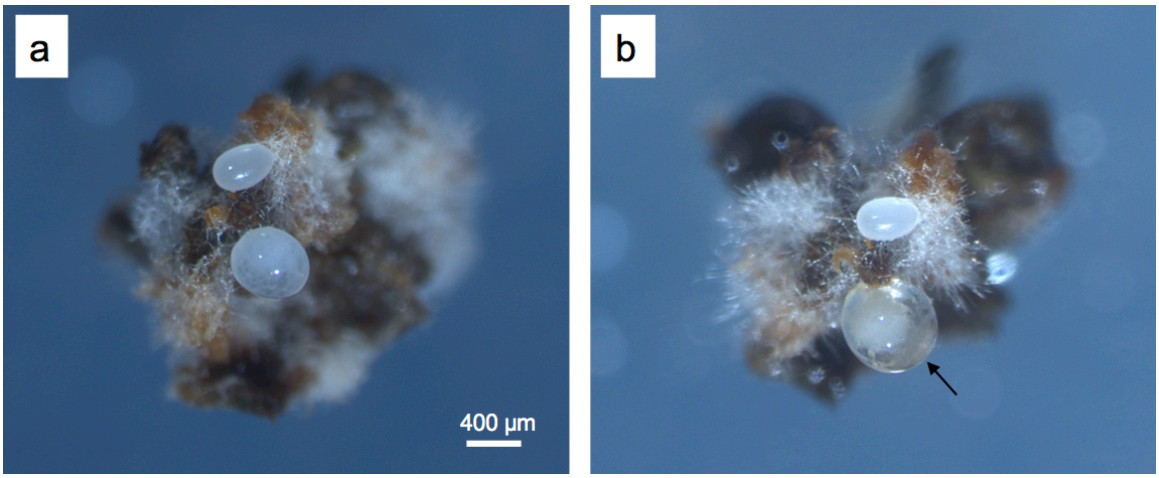

Figure 4.4. a. Experimental setup testing a reproductive egg (R-egg) and a trophic egg (T-egg) placed on a small fragment of fungus garden. b. The T-egg (see arrow) liquified within 24 hours.

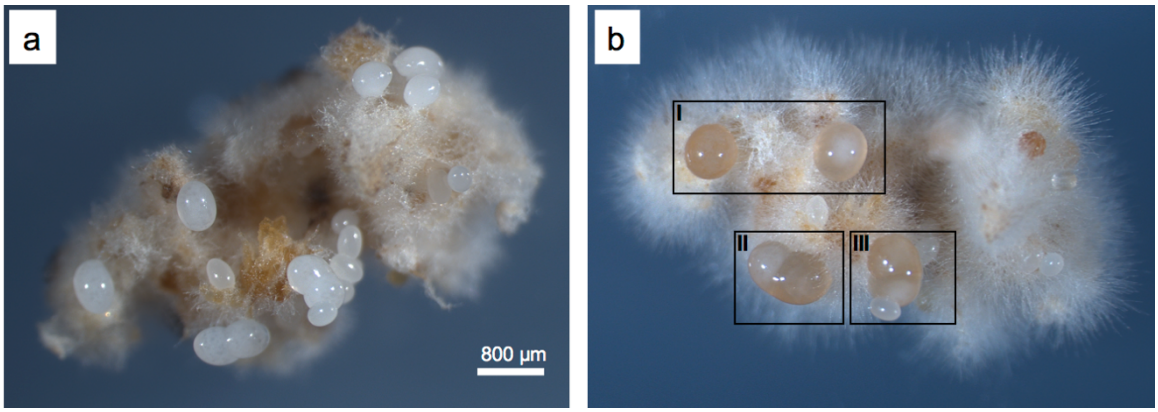

Figure 4.5. a. Experimental setup testing several reproductive eggs (R-eggs) and several trophic eggs (T-eggs) placed on a small fragment of fungus garden. T- eggs were placed on the garden either in isolation (without touching any other eggs, but resting on the fungus garden) or placed such that T-eggs touched other eggs. b. Touching other eggs was not necessary for T-eggs to liquify, and T-eggs in isolation and those touching other eggs both liquified within 24 hours.

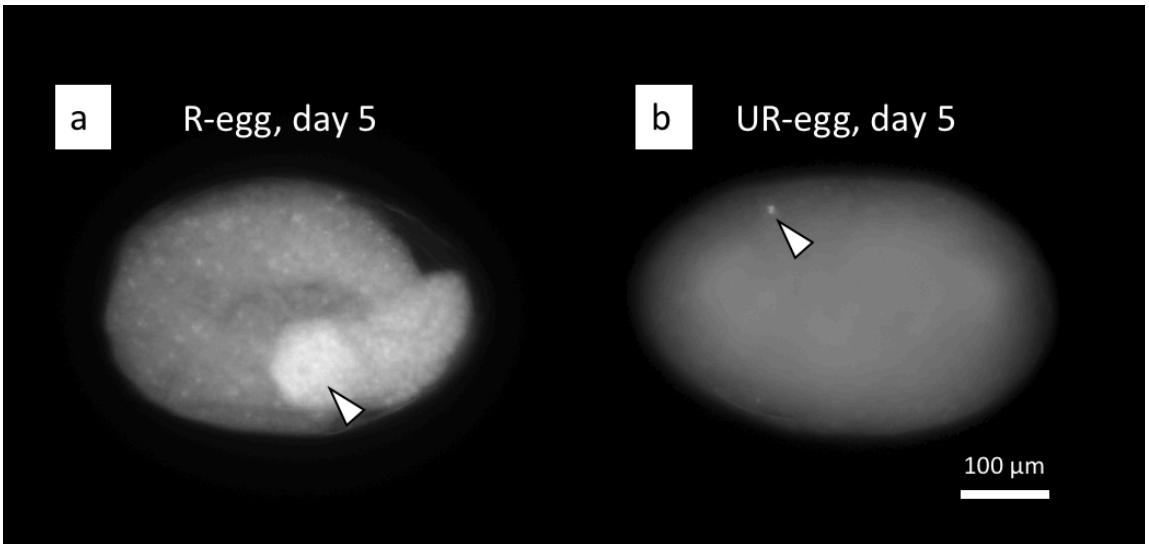

Figure 4.6. Comparison of a reproductive egg (R-egg) and an undeveloped reproductive egg (UR-egg) five days after oviposition by an *Atta texana* foundress. a. The developing ant embryo growing by germ band extension in the R-egg. The arrow indicates the region with a high density of nuclei that will develop into the head lobe. b. Only a single nucleus and no developing embryo are visible in an UR-egg of comparable age (5 days).

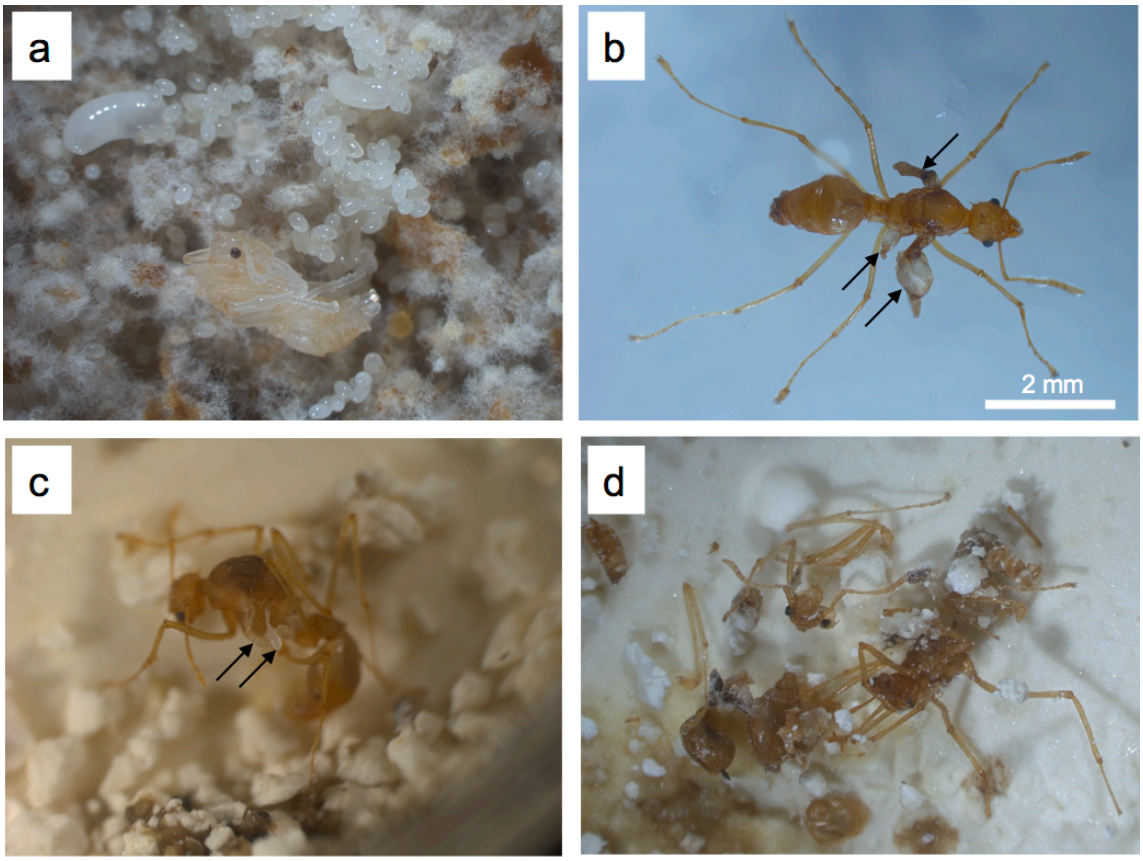

Figure 4.7. Nanitic males produced by an *Atta texana* foundress. Only one foundresses of a total of six foundresses with offspring produced such nanitic males; the other five foundresses raised a typical brood of only workers. a. A nanitic male pupa and larvae and eggs resting on the incipient fungus garden. b. A nanitic male with three stubby wings that failed to expand (see arrows). c. A nanitic male with two stubby wings (see arrows) that was later attacked and killed by the *At. texana* foundress. d. Two nanitic males that had been killed and dismembered by the *At. texana* foundress within 48 hours after the males' eclosion.

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Appendices

APPENDIX A

Table A1. Protocol of whole-mount *in situ* hybridization.

Day 1 proteinase K digestion and postfixation. To rehydrate the fixed samples, we treated the eggs in 75%, 50%, and 25% MeOH (diluted with 0.1% PBST) for 5 minutes under room temperature respectively. Eggs were then washed 3 times with 0.1% PBST (5 minutes each wash) on the shaker (100rpm) under room temperature. The eggs were incubated in proteinase K for 7 minutes, then we stopped the proteinase K digestion by incubating the eggs in 1X glycine for 5 minutes. Glycine solution should always be prepared freshly before use. To decrease the background of the staining images, we washed the eggs twice with 0.1M trimethanolamine and twice with acetyl anhydride in 0.1M trimethanolamine (each wash took 5 minutes). Eggs were then then transferred into 0.1% PBST and be washed 5 times (each wash took 5 minutes). We moved the eggs into a new 1.5mL eppendorf tube with the fixation solution (400uL PEM, 60uL 37% formaldehyde, and 500uL Heptane), then had the samples rocked for 25 minutes under 180rpm. Eggs were then then washed 1 time by 1X glycine and 5 times by 0.1% PBST (each wash took 5 minutes).

Day 1 hybridization

We applied the hybridization solution to the eggs from this step (the recipe for 50ml hybridization solution is: 25ml 100% formamide, 12.5mL 20X SSC (pH 6.5), 250uL salmon sperm (10mg/mL), 250mg torula yeast (10mg/mL), 150uL heparin (50mg/mL), 500uL 10% Tween-20, and 50mL H_2O). The eggs were washed in 50% hybridization solution diluted with 0.1% PBST for 10 minutes. We then washed the eggs

in 100% hybridization solution (prewarmed to 37°C) for 10 minutes. To prehybridize the eggs, we treated the sample in denatured hybridization solution (heated under 95°C for 2 minutes, and then placed immediately on ice for 1 minute) at a minimum of 56°C for 1 to 2 hours. We added 2uL *wingless*(*wg*) probe (NCBI reference sequence: XM_018204929.1) stock to 100uL hybridization solution. The probe needed to be denatured in hybridization solution for 2 minutes at 95°C then put immediately on ice for 5 minutes. Usually, 2uL of the probe should give good staining, but we could add 1 to 5uL probe per 100uL hybridization solution depending on the strength of the probe. We pre-warmed the probe to hybridization temperature, and then removed the prehybridization solution leaving embryos slightly covered to avoid destroying them, as they are very sensitive at high temperatures. Finally, we added the denatured probe and hybridization solution to the eggs. The samples were mixed very gently by swirling, and then were incubated overnight at 56°C.

Day 2 washes

All the following washes were done in a minimum of 56°C water bath, and wash solutions were pre-warmed to 56°C. The eggs were washed embryos twice in the hybridization solution under 56°C for 20 minutes. We then washed the eggs in 1) 3 parts hybridization: 1 part PBTriton, 2) 1 parts hybridization: 1 part PBTriton, and 3) 1 part hybridization: 3 parts PBTriton for 20 minutes respectively. We had samples equilibrate at room temperature for a few minutes. Eggs were then rinsed 5 times with PBTriton at room temp on rocker (each wash took 5 minutes). We also rinsed the eggs 5 times with 0.1% PBST at room temp on rocker (each wash took 5 minutes). The eggs were then blocked in normal goat serum on a rocker at room temperature for 1 hour. Finally, we

incubated the washed eggs in DIG-AP (or Streptavadin) 1:2000 in 0.01% PBST for 2hours at room temperature or overnight at 4°C on the rocker.

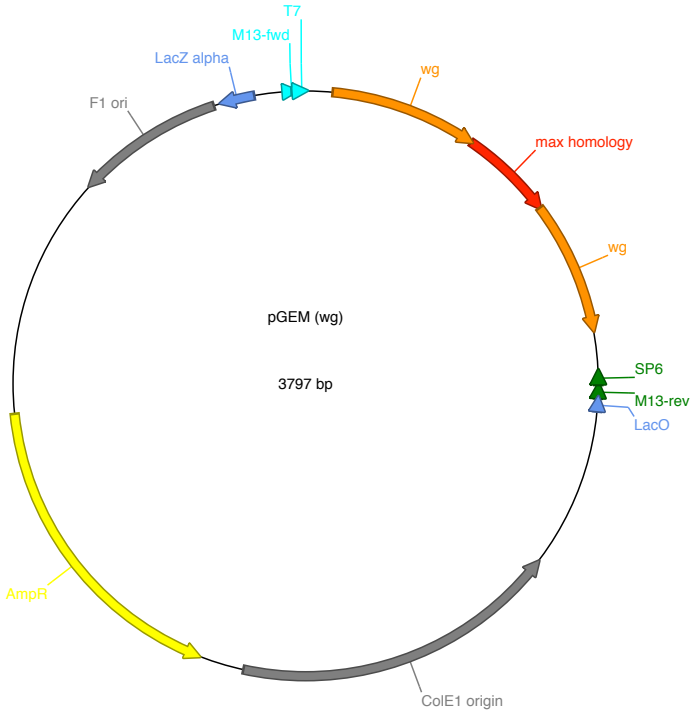
Day 3 staining

We applied the staining buffer to the eggs at the third day (the recipe for 50mL staining buffer is: 2mL NaCl (2.5M), 2.5mL $MgCl_2$ (1M), 5mL Tris-Cl (1M, Ph 9.5), 500uL 10% Tween-20, 50mL H_2O, BCIP (50mg/mL in 100% dimethylformamide [DMF]), and NBT (75mg/mL in 70% DMF/H_2O). The eggs were washed 4 times in PBST at room temperature (each wash took 30 minutes). We then washed the eggs in Tris HCl (pH 9.5) for 5 minutes. To stain the samples, we washed the eggs in staining buffer 3 times (each wash took 5 minutes). The eggs were moved to a welled plate and we removed as much staining buffer without desiccating them. We added 4.5uL NBT (75 mg/ml in 70% DMF/H2O) and 3.5uL BCIP (50mg/ml in 100% dimethylformamide [DMF]) per 1 ml Staining buffer (note: the buffer is light sensitive) and then added to the eggs. The eggs were stained in the dark in welled plate with 600ul staining buffer, NBT, and BCIP. The color usually develops within 30-60 min, and we checked it every fifteen minutes for the first hour, then every 30min-1h although it can be left for up to 48 hrs. The staining buffer can be replaced after several hours if it is needed. To stop the staining reaction, the eggs were rinsed with PBT for 3 to 4 times. We then treated the eggs with 50%PBT/50%MeOH mixture on shaker for 5 minutes, and we treated 100% MeOH to the eggs for 30 minutes on the shaker (MeOH in this step will be discarded after 30 minutes). The samples were treated with fresh 100% MeOH overnight on a shaker. We washed the eggs with 50%PBTw/50%MeOH on shaker for 5 minutes, and then the eggs were washed with PBT for 3 hours. Since the eggs would be mounted in 70% Glycerol (diluted with 0.1% PBST) at the end, we treated the samples with different concentrations

of glycerol gradually as the following steps: 1) 30% Glycerol for 5 minutes, 2) 50% glycerol for 30 minutes, and 3) 70% glycerol for 3 hours (can stay in 4 degree overnight). We took images of the mounted eggs.

APPENDIX B

Table B1. Plasmid for amplifying *Atta colombica* Wnt-1 mRNA. We designed the *wingless* probe based on the sequence of that gene in the published genome of *Atta colombica* (Wnt-1 mRNA; NCBI reference sequence: XM_018204929.1). The sequence of wingless mRNA (orange and red regions) is listed below.



Sequence of *wingless* mRNA:

TTTTTACCGATGTCAGCAGGTTCCATACACATGGAACCGGTCTACGCGACCCT
GAGAAGAAAGCAGAGGAGGCTCGCTAGAGAAAATCCGGGAGTGCTGATGGC
AGTATCAAGGGGTGCGAACCAGGCTATCGCCGAATGCCAATATCAGTTTCGC
AACCGGCGATGGAATTGCTCGACGAAAAATTTTCTTAGAGGGAAAAATCTCT
TCGGCAAGATCGTCGACAGAGGCTGTCGAGAGACCGCTTTTATCTACGCCAT
CACGAGTGCGGCAGTGACTCACAGTATCGCTAGGGCATGTAGCGAAGGCAGC
ATTCAGTCGTGTTCCTGCGATTACACTCACCAATCACACGCATCATCTGCCGT
TCGAGATTGGGAATGGGGTGGTTGCTCGGATAACATTGGATACGGCTTCAAG
TTCTCTCGCGAATTCGTTGATACTGGCGAACGTGGCCGGAATCTTCGCGAGAA
GATGAATCTACATAACAATGAGGCCGGCAGAGCCCACGTGACTTCGGAGATG
CGTCAGGAATGCAAGCGCCACGGCATGTCCGGCTCGTGCACGGTAAAGACTT
GCTGGATGCGGTTGCCGAATTTCCGCGTGGTCGGCGATAACCTGAAAGATCG
CTTCGATGGGGCTTCTCGGGTAATGGTCAGTAATTCAGACCGTGCACGCGTTA
ACAACAATGCCATTACCAGCAATTCGGCGAGCAATTCCGTGCACCAGCACCG
TGAGGGTCTCGGACGCCGGCAACGCTACAATTTCCAGCTGAAGCCATACA

APPENDIX C

Table C1. R-script for Chapter 3 Experiment 1-5, shown graphically in Figures 3.1-3.7. Pairwise comparison analyses are shown in Table 3.1-3.6.

```
# the packages that used in this R script
# use install.packages() to install them in R
library(lme4)
library(nlme)
library(tidyverse)
library(ggplot2)

# for pairwise comparison in linear mixed model
library(emmeans)

#### FIG 3.1 ####
# read-in your data
# make sure to have the script and the .csv file at the same location
# use setwd() to change the directory to the folder
rawdat <- read.table(file = "TableS1a_Exp1_Fig1.csv", sep = ",", header
 = TRUE, fill = TRUE)

# preping function for calculating SE
Se_Fun <- function (dat){
  means = c()
  for (i in 1:10000){
    means = c(means, mean(sample(dat, length(dat), replace = TRUE)))
  }
  sd(means)
}

# the raw data had transformed into long format
dat_long <- rawdat

# plotting
dat_sum <- dat_long %>%
  group_by(trmt, week) %>%
  summarize(weight_avg = mean(weight),
            se = Se_Fun(weight))

dat_sum$trmt <- factor(dat_sum$trmt, levels = c("0Q30W", "1Q30W","5Q30W
","10Q30W"))
pd <- position_dodge(width = 0.7)
dat_sum %>%
  subset(weight_avg != "0") %>%
  ggplot(aes(x = week, y = weight_avg, group = trmt)) +
```

```
  geom_point(aes(color = trmt), size = 2) +
  geom_errorbar(aes(ymin = weight_avg - se, ymax = weight_avg + se, col
or = trmt), width = 0.1, position = pd) +
  geom_line(aes(color = trmt), alpha = 0.7) +
  labs(x="Week", y="Average fungus weight (g)") +
  coord_cartesian(ylim = c(0,0.4),
                  xlim = c(0,31)) +
  scale_x_continuous(breaks = seq(0,31,5)) +
  scale_color_manual(labels = c("0Q-30W","1Q-30W","5Q-30W","10Q-30W"),
                     values = c("gray60", "#bf5700", "steelblue3", "gra
y0"))+
  theme(legend.position = c(0.9, 0.8),
        legend.title = element_text("treatment"),
        legend.direction = "vertical",
        panel.grid.major =  element_blank(), panel.grid.minor = element
_blank(),
        panel.background =  element_blank(), axis.line = element_line(c
olour = "grey"))

# run linear mixed model
# week was treated as the random effect
mod <- lme(weight ~ trmt, random = ~1|week, data = dat_long)
summary(mod)

# pairwise comparison
emmeans(mod, list(pairwise ~ trmt), adjust = "tukey")

#### FIG 3.2 ####
# read-in your data
rawdat <- read.table(file = "TableS2a_Exp1_Fig2.csv", sep = ",", header
 = TRUE, fill = TRUE)

# preping function for calculating SE
Se_Fun <- function (dat){
  means = c()
  for (i in 1:10000){
    means = c(means, mean(sample(dat, length(dat), replace = TRUE)))
  }
  sd(means)
}

# transforming the raw data into long format
dat_long <- rawdat %>%
  subset(Queen != "0Q") %>%
  gather(week, weight, -c(Queen, Worker, trmt, yr)) %>%
  subset(weight != "NA") %>%
```

```
  mutate(week = substr(week, 2, 3))

## plotting
dat_long$trmt_reord <- factor(dat_long$trmt, levels = c("1Q30W", "5Q30W
","10Q30W"))

dat_sum <- dat_long %>%
  group_by(trmt, week) %>%
  summarize(weight_avg = mean(weight),
            se = Se_Fun(weight))

dat_sum %>%
  ggplot(aes(x = week, y = weight_avg, group = trmt)) +
  geom_point(aes(color = trmt), size = 2) +
  geom_errorbar(aes(ymin = weight_avg - se, ymax = weight_avg + se, col
or = trmt), width = 0.1) +
  geom_line(aes(color = trmt)) +
  labs(x="Week", y="Average fungus weight (g)") +
  facet_wrap(~trmt)

# figure
dat_sum$trmt <- factor(dat_sum$trmt, levels = c("1Q30W","5Q30W","10Q30W
"))
pd <- position_dodge(width = 0.25)
FIG2 <- dat_sum %>%
  ggplot(aes(x = week, y = weight_avg, group = trmt)) +
  geom_point(aes(color = trmt), size = 2) +
  geom_errorbar(aes(ymin = weight_avg - se, ymax = weight_avg + se, col
or = trmt), width = 0.1, position = pd) +
  geom_line(aes(color = trmt)) +
  labs(x="Week", y="Average fungus weight (g)") +
  coord_cartesian(ylim = c(0,0.5)) +
  scale_color_manual(labels = c("1Q-30W","5Q-30W","10Q-30W"),
                     values = c("#bf5700", "steelblue3", "gray0")) +
  theme(legend.position = c(0.9, 0.8),
        legend.title = element_text("treatment"),
        legend.direction = "vertical",
        panel.grid.major =  element_blank(), panel.grid.minor = element
_blank(),
        panel.background =  element_blank(), axis.line = element_line(c
olour = "grey"))

# change the x axis scale labels
FIG2 + scale_x_discrete(breaks=c("01","02","03","04","05","06","07","08
","09","10","11","12","13"),
                        labels=c("1","2","3","4","5","6","7","8","9","1
```

```
0","11","12","13"))

# run linear mixed model
# week was treated as the random effect
mod <- lme(weight ~ trmt, random = ~1|week, data = dat_long)
summary(mod)

# pairwise comparison
emmeans(mod, list(pairwise ~ trmt), adjust = "tukey")

#### FIG 3.3 ####
# read-in your data
rawdat <- read.table(file = "TableS3a_Exp2_Fig3_FigS1.csv", sep = ",",
header = TRUE, fill = TRUE)

# preping function for calculating SE
Se_Fun <- function (dat){
  means = c()
  for (i in 1:10000){
    means = c(means, mean(sample(dat, length(dat), replace = TRUE)))
  }
  sd(means)
}

# transforming the raw data into long format
dat_long <- rawdat %>%
  subset(yr != 17) %>%
  subset(Queen != "0Q") %>%
  gather(week, weight, -c(Queen, Worker, trmt, yr)) %>%
  subset(weight != "NA") %>%
  mutate(week = substr(week, 2, 3))

# plotting
dat_sum <- dat_long %>%
  group_by(trmt, week) %>%
  summarize(weight_avg = mean(weight),
            se = Se_Fun(weight))

# ExpIII figure # It works! No facet!
pd <- position_dodge(width = 0.25)
FIG3 <- dat_sum %>%
  ggplot(aes(x = week, y = weight_avg, group = trmt)) +
  geom_point(aes(color = trmt), size = 2) +
  geom_errorbar(aes(ymin = weight_avg - se, ymax = weight_avg + se, col
or = trmt), width = 0.1, position = pd) +
  geom_line(aes(color = trmt)) +
```

```
  labs(x="Week", y="Average fungus weight (g)") +
  coord_cartesian(ylim = c(0,0.6))+
  scale_color_manual(labels = c("1Q-30W","1Q-60W","1Q-90W"),
                     values = c("#bf5700", "darkolivegreen4", "gray30")
) +
  theme(legend.position = c(0.9, 0.8),
        legend.title = element_text("treatment"),
        legend.direction = "vertical",
        panel.grid.major =  element_blank(), panel.grid.minor = element
_blank(),
        panel.background =  element_blank(), axis.line = element_line(c
olour = "grey"))

# change the x axis scale labels
FIG3 + scale_x_discrete(breaks=c("01","02","03","04","05","06","07","08
","09","10","11","12","13"),
                        labels=c("1","2","3","4","5","6","7","8","9","1
0","11","12","13"))

# run linear mixed model
# week and year were treated as the two random effects
mod1 <- lmer(weight ~ trmt + (1|week) + (1|yr), data = dat_long)
summary(mod1)

# when using lmer(), we need package "lmerTest" to help us finding the
p-value
install.packages("lmerTest")
library(lmerTest)
lmm <- lmer(weight ~ trmt + (1|week) + (1|yr), data = dat_long)
summary(lmm)
anova(lmm)

# pairwise comparison
emmeans(lmm, list(pairwise ~ trmt), adjust = "tukey")

#### FIG 3.4 ####
# read-in your data
rawdat <- read.table(file = "TableS4a_Exp3_Fig4.csv", sep = ",", header
 = TRUE, fill = TRUE)

# preping function for calculating SE
Se_Fun <- function (dat){
  means = c()
  for (i in 1:10000){
    means = c(means, mean(sample(dat, length(dat), replace = TRUE)))
  }
```

```r
  sd(means)
}

# transforming the raw data into long format
dat_long <- rawdat %>%
  gather(week, weight, -c(Queen, Worker, trmt, yr)) %>%
  subset(weight != "NA") %>%
  mutate(week = substr(week, 2, 3))

# plotting
dat_sum <- dat_long %>%
  group_by(trmt, week) %>%
  summarize(weight_avg = mean(weight),
            se = Se_Fun(weight))

dat_sum$trmt <- factor(dat_sum$trmt, levels = c("1Q6W","1Q18W","1Q60W",
 "1Q90W"))

# figure
pd <- position_dodge(width = 0.25)
FIG4 <- dat_sum %>%
  ggplot(aes(x = week, y = weight_avg, group = trmt)) +
  geom_point(aes(color = trmt), size = 2) +
  geom_errorbar(aes(ymin = weight_avg - se, ymax = weight_avg + se, col
or = trmt), width = 0.1, position = pd) +
  geom_line(aes(color = trmt)) +
  labs(x="Week", y="Average fungus weight (g)") +
  scale_color_manual(labels = c("1Q-6W","1Q-18W","1Q-60W","1Q-90W"),
                     values = c("snow4","lightblue3","darkolivegreen4",
 "gray30")) +
  coord_cartesian(ylim = c(0,0.9))+
  theme(legend.position = c(0.9, 0.9),
        legend.title = element_text("treatment"),

        legend.direction = "vertical",
        panel.grid.major =  element_blank(), panel.grid.minor = element
_blank(),
        panel.background =  element_blank(), axis.line = element_line(c
olour = "grey"))

# change the x axis scale labels
FIG4 + scale_x_discrete(breaks=c("01","02","03","04","05","06","07","08
","09","10","11","12","13"),
                        labels=c("1","2","3","4","5","6","7","8","9","1
0","11","12","13"))
```

```r
# run linear mixed model
# week was treated as the random effect
mod <- lme(weight ~ trmt, random = ~1|week, data = dat_long)
summary(mod)

# pairwise comparison
emmeans(mod, list(pairwise ~ trmt), adjust = "tukey")

#### FIG 3.5 ####
# read-in your data
rawdat <- read.table(file = "TableS5a_Exp4_Fig5.csv", sep = ",", header
 = TRUE, fill = TRUE)

# preping function for calculating SE
Se_Fun <- function (dat){
  means = c()
  for (i in 1:10000){
    means = c(means, mean(sample(dat, length(dat), replace = TRUE)))
  }
  sd(means)
}

# transforming the raw data into long format
dat_long <- rawdat %>%
  gather(week, weight, -c(Queen, Worker, trmt, yr)) %>%
  subset(weight != "NA") %>%
  mutate(week = substr(week, 2, 3))

# plotting
dat_sum <- dat_long %>%
  group_by(trmt, week) %>%
  summarize(weight_avg = mean(weight),
            se = Se_Fun(weight))

dat_sum$trmt <- factor(dat_sum$trmt, levels = c("1Q60W_0.1","1Q60W_0.45
","1Q90W_0.1", "1Q90W_0.45"))

# figure
pd <- position_dodge(width = 0.25)
FIG5 <- dat_sum %>%
  ggplot(aes(x = week, y = weight_avg, group = trmt)) +
  geom_point(aes(color = trmt), size = 2) +
  geom_errorbar(aes(ymin = weight_avg - se, ymax = weight_avg + se, col
or = trmt), width = 0.1, position = pd) +
  geom_line(aes(color = trmt)) +
  labs(x="Week", y="Average fungus weight (g)") +
```

```
  scale_color_manual(labels = c("1Q-60W + 0.1g fungus ","1Q-60W + 0.45g
 fungus","1Q-90W + 0.1g fungus","1Q-90W + 0.45g fungus"),
                     values = c("darkolivegreen4","darkseagreen3","gray
30", "gray60")) +
  coord_cartesian(ylim = c(0,0.9))+
  theme(legend.position = c(0.85, 0.9),
        legend.title = element_text("treatment"),
        legend.direction = "vertical",
        panel.grid.major =  element_blank(), panel.grid.minor = element
_blank(),
        panel.background =  element_blank(), axis.line = element_line(c
olour = "grey"))

# change the x axis scale labels
FIG5 + scale_x_discrete(breaks=c("01","02","03","04","05","06","07","08
","09","10","11","12","13"),
                        labels=c("1","2","3","4","5","6","7","8","9","1
0","11","12","13"))

# run linear mixed model
# week and year were treated as the two random effects
mod1 <- lmer(weight ~ trmt + (1|week) + (1|yr), data = dat_long)
summary(mod1)

# when using lmer(), we need package "lmerTest" to help us finding the
p-value
install.packages("lmerTest")
library(lmerTest)
lmm <- lmer(weight ~ trmt + (1|week) + (1|yr), data = dat_long)
summary(lmm)
anova(lmm)

# pairwise comparison
emmeans(lmm, list(pairwise ~ trmt), adjust = "tukey")

#### FIG 3.6 ####
# read-in your data
rawdat <- read.table(file = "TableS6a_Exp5_Fig6.csv", sep = ",", header
 = TRUE, fill = TRUE)

# plotting
ggplot(eggraw, aes(x=EggNum, fill=Pool))+
  geom_histogram(binwidth = 0.5, alpha = 0.8, position = "dodge")+
  labs(y = "Number of queens",
       x = "Number of eggs laid by a single queen within 24 hours",
       fill = "Replicate run") +
```

```
  coord_cartesian(ylim = c(0,50))+
  scale_x_continuous(breaks = seq(0, 5, 1))+
  scale_fill_manual(values = c("navajowhite", "tan1", "#bf5700"))+
  theme(legend.position = c(0.8, 0.8),
        legend.direction = "horizontal",
        panel.grid.major =  element_blank(), panel.grid.minor = element
_blank(),
        panel.background =  element_blank(), axis.line = element_line(c
olour = "grey"))

#ANOVA
model <- aov(EggNum ~ Pool, data=eggraw)
anova(model)
summary(model)

#Analaysis
attributes(model)
model$coefficients
TukeyHSD(model)
plot(TukeyHSD(model), las=1)

#### FIG 3.7 ####
# read-in your data
rawdat <- read.table(file = "TableS3a_Exp2_Fig3_FigS1.csv", sep = ",",
header = TRUE, fill = TRUE)

# preping function for calculating SE
Se_Fun <- function (dat){
  means = c()
  for (i in 1:10000){
    means = c(means, mean(sample(dat, length(dat), replace = TRUE)))
  }
  sd(means)
}

# transforming the raw data into long format
# only select the 2018 data
dat_long <- rawdat %>%
  filter(yr == "18") %>%
  subset(Queen != "0Q") %>%
  gather(week, weight, -c(Queen, Worker, trmt, yr)) %>%
  subset(weight != "NA") %>%
  mutate(week = substr(week, 2, 3))

# plotting
dat_sum <- dat_long %>%
```

```r
  group_by(trmt, week) %>%
  summarize(weight_avg = mean(weight),
            se = Se_Fun(weight))

# figure
pd <- position_dodge(width = 0.25)
FIGS1 <- dat_sum %>%
  ggplot(aes(x = week, y = weight_avg, group = trmt)) +
  geom_point(aes(color = trmt), size = 2) +
  geom_errorbar(aes(ymin = weight_avg - se, ymax = weight_avg + se, col
or = trmt), width = 0.1, position = pd) +
  geom_line(aes(color = trmt)) +
  labs(x="Week", y="Average fungus weight (g)") +
  coord_cartesian(ylim = c(0,0.6))+
  scale_color_manual(labels = c("1Q-30W","1Q-60W","1Q-90W"),
                     values = c("#bf5700", "darkolivegreen4", "gray30")
) +
  theme(legend.position = c(0.9, 0.8),
        legend.title = element_text("treatment"),
        legend.direction = "vertical",
        panel.grid.major =  element_blank(), panel.grid.minor = element
_blank(),
        panel.background =  element_blank(), axis.line = element_line(c
olour = "grey"))

# change the x axis scale labels
FIGS1 + scale_x_discrete(breaks=c("01","02","03","04","05","06","07","0
8","09","10","11","12","13"),
                         labels=c("1","2","3","4","5","6","7","8","9"
,"10","11","12","13"))

# run linear mixed model
# week was treated as the random effect
mod <- lme(weight ~ trmt, random = ~1|week, data = dat_long)
summary(mod)

# pairwise comparison
emmeans(mod, list(pairwise ~ trmt), adjust = "tukey")
```

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Vita

Chi-Chun (Andy) Fang (方棋羣) was born in Taipei, Taiwan. He grew up in Sanxia District, a place full of animals and natural scenery. In his childhood, he developed keen interests in reading and a strong passion for Biology. After graduating from Hsin Tien Senior High School, he started his entomology and biology career at National Taiwan University (NTU) in 2003. In college, he managed to find the balance between school work and NTU chorus and received 5 times the NTU Presidential Award. In 2006, he received the College Student Research Training Fellowship from the Ministry of Science and Technology, and he studied the life-history traits of the invasive mealybug, *Phenacoccus solenopsis* Tinsley, in the lab of Dr. Wen-Jer Wu. After receiving his Bachelor of Science degree from NTU in 2007, he was enrolled in the Master program at NTU under the supervision of Dr. Wen-Jer Wu and Dr. Ju-Chun Hsu. He studied population dynamics of the oriental fruit fly, *Bactrocera dorsalis*, and received his Master of Science degree from NTU in 2009. In 2010, he completed his compulsory military service as a corporal of the Taiwan army. During the following year, he devoted himself to preparations for graduate program applications. In 2012, he interviewed with his future advisor in Okinawa, Japan and started his Ph. D. program at The University of Texas at Austin under the supervision of Dr. Ulrich G. Mueller.

Address: ccfang@utexas.edu
This dissertation was typed by the author.